GENETIC VARIATION AND POPULATION GENETIC STRUCTURE OF MUSKRAT, *ONDATRA ZIBETHICUS*, AT DIFFERENT SPATIAL SCALES

by

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ABSTRACT

Understanding the factors and processes that influence intraspecific genetic variation are essential to better understand evolutionary processes. In this research, I examined patterns of gene flow and their effects on the distribution of genetic variation and spatial genetic structuring at different spatial scales. I used a combination of population genetics, spatial analysis, morphometrics and phylogeography in order to understand the patterns of genetic variation and their resulting phenotypic variations in a semi-aquatic species, the muskrat (*Ondatra zibethicus*).

In order to investigate intraspecific genetic variations, I isolated and characterized 12 polymorphic microsatellite loci. These neutral genetic markers were highly polymorphic and presented moderate to high levels of genetic variability which make them useful for the study of contemporary population genetic structure and patterns of gene flow. Some of these microsatellite loci amplified in several rodent species and may be useful in the study of patterns of genetic variation in these species. The results from the cross-amplification confirmed the higher success of amplification in species more closely related to the muskrat.

At a fine spatial scale, I assessed the influence of landscape features on patterns of gene flow and population genetic structure in muskrat among three watersheds near Sudbury, Ontario, Canada. Contrary to my hypothesis, the landscape heterogeneity did not prevent dispersal. A single genetic cluster was identified and no genetic differences were detected among the watersheds as a result of high levels of gene flow. Using a least cost path (LCP) approach, I found a positive relationship between individual pairwise genetic distances and least cost distances when roads were considered as corridors for movements.

The results of this research also indicated that open landscapes and urban areas (excluding roads) seemed to restrict but not prevent gene flow within the study area. These findings underline the high dispersal ability of generalist species in their use of landscape for movement and highlight how landscape features often considered barriers to animal movements are corridors for other species.

Patterns of gene flow and population genetic structure may be affected by extrinsic and intrinsic factors. The resulting genetic divergence may ultimately lead to phenotypic differentiation. At a large spatial scale, I examined population genetic structure in muskrat and assessed phenotypic differences between the identified genetic clusters. I investigated phenotypic variation by measuring differences in skull size and shape using geometric morphometrics. The results indicated four to five distinct genetic clusters with muskrat from Manitoulin Island and from southern townships being genetically different from the other regions due to the presence of physical barriers. Although the presence of moderate spatial genetic structuring due to major physical barriers supports my hypothesis, I detected relatively high levels of gene flow at this spatial scale. Contrary to my prediction, I found that only some genetic clusters, such as Manitoulin Island, presented phenotypic divergence for the shape of the skull. These phenotypic variations may be due to variation in masticatory muscles as a result of different feeding ecologies. The association of genetic divergence with phenotypic variation in some regions may suggest the presence of genetic drift or local adaptation.

Finally, at a macrogeographic scale, contemporary and historical processes play an important role in shaping the distribution of genetic variation. Spatial genetic structuring may ultimately lead to population divergence. Divergent populations may represent different subspecies and patterns of genetic structure should reflect these subspecies units.

In order to assess the relationship between spatial genetic structuring and subspecies groupings, I examined the contemporary population genetic structure of muskrat and its concordance with four geographically distinct subspecies designations across northern North America. I detected a strong pattern of isolation by distance and the presence of spatial genetic structuring among western regions, eastern regions and Newfoundland (NF). Contrary to my prediction, the patterns of genetic structure did not reflect subspecies groupings. The results of this study indicated no differentiation between *Ondatra zibethicus spatulus* (northwest) and *O. z. albus* (central), but they suggested a distinction between *O. z. obscurus* (NF) and *O. z. zibethicus* (east). This study highlights the need for more phylogenetic studies in order to better understand intraspecific divergence and the genetic characterization of subspecies.

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CHAPTER 1. General introduction

Background

Population genetics examines the amount and distribution of genetic variation within and among populations, and includes the study of intraspecific genetic diversity, genetic differentiation and genetic distance (Lowe et al. 2004; Hedrick 2005). In many species, naturally occurring populations are structured into smaller units. This subdivision may be the result of environmental factors as well as biological ones and the pattern and degree of population structuring will be affected by the degree of gene flow between the subpopulations (Hedrick 2005). Studying patterns of dispersal and gene flow is essential to understand genetic variation among and within populations. Gene flow, the movement of immigrant genes into a population, is affected by both extrinsic and intrinsic factors (Lowe et al. 2004). Biological factors such as population size, vagility and social structure may influence patterns of gene flow and therefore the extent of structuring (Hedrick 2005). Demographic parameters have also been shown to affect gene flow and lead to homogenization or structuring of populations (Berthier et al. 2005; Gauffre et al. 2008). These factors are dependent on the geographical and historical contexts of populations (Lougheed et al. 1999; Lowe et al. 2004). Environmental factors such as physical barriers and landscape structure have a strong influence on gene flow and population genetic structure (Rueness et al. 2003; Lowe et al. 2004; Trizio et al. 2005; Vandergast et al. 2007). The importance of landscape structure on the patterns of gene flow between populations has been shown for several animal taxa including invertebrates (Vandergast et al. 2007; Worthington Wilmer et al. 2008), fishes (Leclerc et al. 2008; Faulks et al. 2011), amphibians (Spear et al. 2005; Mullen et al. 2010), reptiles (Row et al. 2010; Klug et al. 2011), birds (Hull et al. 2008a; Pavlacky et al. 2009) and mammals (Broquet et al. 2006;

Garroway et al. 2011). These landscape features include historical landscape discontinuities such as barriers created by the presence of ice sheets and glacial refugia during the Pleistocene (Durka et al. 2005; Margraf et al. 2007) as well as contemporary landscape discontinuities (Berthier et al. 2005; Johansson et al. 2006). Recent events such as anthropogenic disturbances may decrease connectivity among populations (Cushman 2006; Riley et al. 2006). Understanding the extent and patterns of gene flow will help predict how changes in the environment may affect genetic variation across natural populations.

The landscape genetics approach has been developed in an attempt to relate population genetic structure and gene flow to landscape characteristics (Manel et al. 2003; Storfer et al. 2007). It has facilitated the understanding of spatial genetic structure (Coulon et al. 2004; Berthier et al. 2005; Vignieri 2005; Klug et al. 2011). This recent field employs both landscape ecology and population genetics in order to describe spatial genetic patterns and to determine how landscape characteristics influence these patterns (Manel et al. 2003). Population genetic structure, particularly in fast changing environments, can be understood by studying landscape characteristics that impede movements and hence dispersal. The rapid expansion of human population and activities (including agriculture, logging, industrialization, urbanization) leads to landscape alteration (Lindenmayer and Fischer 2006). As a result of anthropogenic disturbances or natural events, habitat loss and fragmentation lead to a heterogeneous landscape that may disrupt animal movements and dispersal. Landscape alterations are also expected to decrease habitat connectivity which seems to be one of the main restricting factors for animal dispersal (Taylor et al. 1993; Goodwin and Fahrig 2002). Landscape connectivity between optimal habitats allows for dispersal and gene flow among populations (Goodwin and Fahrig 2002). However, even in unfragmented landscapes, landscape features may affect connectivity and prevent gene

flow (Cushman 2006). Numerous studies have examined the effects of ecological barriers on genetic structuring but more recently studies have focused on the effect of habitat connectivity (Coulon et al. 2004; Vignieri 2005; Broquet et al. 2006). Dispersal associated with high levels of gene flow homogenizes genetic variations and reduces population genetic structure. However, when habitat heterogeneity is present due to ecological barriers and loss of habitat connectivity, low levels of gene flow leads to population genetic divergence and population structuring.

Several studies have demonstrated the effects of ecological barriers or landscape characteristics on population genetic structure at different spatial scales (Pope et al. 2006; Angelone et al. 2011). On a microgeographic scale (within the dispersal range of the species) where physical or ecological barriers do not inhibit migration, theory predicts that territorial behavior, nonrandom dispersal of different genotypes, or sedentary life habit may produce spatial heterogeneity in genetic diversity (Lougheed et al. 1999). Species which have the potential to disperse over large distances and have large home ranges are predicted to show little spatial structuring at microgeographic scales (Coulon et al. 2004), whereas species with small home ranges and limited dispersal would be expected to show evidence of spatial structuring (Mossman and Waser 2001; Peakall et al. 2003; Berthier et al. 2005; Vignieri 2005). Moreover, the restriction to a specific environment, such as the aquatic environment, may limit gene flow and therefore lead to strong genetic structure.

The study of dispersal patterns and its extent among populations is possible through indirect measures of gene flow using various molecular markers. Recent progress in genetic technology and analyses has allowed the efficient measurement of gene flow at different temporal and spatial scales. Mitochondrial DNA (mtDNA) has often been used to examine postglacial colonization of species from glacial refugia and to infer signs of past barriers

(Schaschl et al. 2003; Durka et al. 2005; Lee-Yaw et al. 2008). In contrast, because of their high mutation rate, microsatellite DNA loci (nuclear DNA) have been used to characterize genetic differentiation between populations that are the result of more recent events (Pope et al. 2006; Rowe and Beebee 2007). Moreover, advances in the field of landscape genetics have helped in the analyses and our understanding of spatial population genetics. The use of Bayesian methods combined with spatial data acquired through the Geographic Information System (GIS) has helped not only in the understanding of isolation by distance patterns and the identification of barriers but also to better understand structural connectivity affecting gene flow among populations or individuals. Several approaches have been developed in order to quantify this connectivity such as Least Cost Path (LCP) models that have been used over the last 15 years (Adriaensen et al. 2003; McRae et al. 2008) and more recently circuit theory (McRae et al. 2008).

Studies of the effects of current landscape heterogeneity on dispersal and population structure have been primarily focusing on endangered species for conservation purposes (Darvill et al. 2006; Stevens et al. 2006; Funk et al. 2008). However, these threatened species could also benefit from the study of population genetic structure of widespread species (Noël et al. 2007). A widespread native species could be used as an indicator species in order to understand the effects of habitat loss and fragmentation on populations (Vandergast et al. 2007). In the case of invasive species, such as the muskrat in Europe (Zachos et al. 2007) or the green crab *Carcinus maenas* (Roman and Palumbi 2004), understanding colonization and gene flow patterns may help in controlling the expansion of such species. Moreover, most studies on population genetics theory use animal models that are exclusively terrestrial (Mossman and Waser 2001; Rueness et al. 2003; Coulon et al. 2004) or aquatic (Castric et al. 2001; Roman and Palumbi 2004; Primmer et al. 2006).

Several barriers to gene flow have been identified for a variety of organisms in each system. However, in most studies, aquatic and terrestrial environments are considered separately and each of them is viewed as an ecological barrier to the organism that disperses within the other environment. The combination of these two ecosystems adds complexity to the landscape analyzed and few studies have been interested in the effects of this complex landscape on gene flow and population structure of mammals (but see Vignieri 2005, Zalewski et al. 2009 and Carranza et al. 2012). Several studies have examined the effects of landscape composition on amphibians (Funk et al. 2005; Cushman 2006; Goldberg and Waits 2010; Richardson 2012). Although these species are not limited to the waterbodies and riparian corridors for dispersal (Goldberg and Waits 2010; Murphy et al. 2010), numerous barriers to gene flow have been identified (Funk et al. 2005; Cushman 2006; Goldberg and Waits 2010). However, the effect of landscape on gene flow remains species-specific (Cushman 2006; Goldberg and Waits 2010). When considering the two ecosystems (terrestrial and aquatic), the effects of habitat fragmentation and habitat loss in shaping population structuring may differ and factors affecting gene flow and hence structuring may also vary. Semi-aquatic mammals may be affected both by terrestrial landscape features and watershed characteristics for their dispersal and may therefore present different patterns of genetic variation within and among populations than strictly terrestrial species. Although they are dependent on the hydrographic network for movements, they also have the capacity to use terrestrial pathways which may increase connectivity between favorable habitat patches. Therefore, for these semi-aquatic species, population genetic structuring may be more homogeneous than predicted for strictly terrestrial mammals. However, distance between watersheds and the type of landscape may be important factors restricting their dispersal and hence gene flow. Animals inhabiting

such complex environments may thus display unique patterns of gene flow and population genetic structure.

A reduction in gene flow leading to population genetic structuring should ultimately lead to population divergence with respect to phenotype. These phenotypic differences may be the result of evolutionary forces such as local adaptation or genetic drift (Merilä and Crnokrak 2001; Hedrick 2005; Leinonen et al. 2006). When local adaptation occurs, phenotypic differences influence how individuals perform, and individuals better adapted to the environment leave more offspring than those lacking favorable traits which leads to a change in the population (Hedrick 2005; Smith et al. 2005). However, phenotypic divergence can also be the result of genetic drift in which case there are random changes in the gene pool of small populations (Frankham et al. 2002). Genetic drift can further cause allele fixation which leads to a loss of genetic variation (Hedrick 2005).

Studies of phenotypic variation are usually investigated by measuring variation in size and shape. Traditionally, the assessment of these variations has been performed using linear measurements that emphasize variation in size alone. However, the analysis of shape is often more informative. Recent methods of shape analysis have evolved considerably and the use of geometric morphometrics analysis is now frequent in the analysis of morphological variation (Rohlf 1999; Adams et al. 2004). This method allows the separation of the shape from the size and requires the use of landmarks (Rohlf and Marcus 1993; Adams et al. 2004). In vertebrates, and more specifically in mammals, the study of population divergence with respect to phenotype has often been performed through the examination of the skull morphology as a measure of phenotypic variation. This phenotypic trait is a complex structure influenced by functional and environmental factors which reflects different selective pressures. Variations in the species habitat may lead to

differences in life habits and may be reflected in the skull morphology (Grieco and Rizk 2010).

Restricted gene flow may lead to intraspecific population structuring and ultimately population divergence (genetic and phenotypic) which is often the precursor to speciation. Significant genetic differentiation associated with phenotypic variations has been used to characterize subspecies (Johnsen et al. 2006; Phillimore and Owens 2006; Hull et al. 2008b). Traditionally, subspecies were defined mainly based on morphological characteristics (Phillimore and Owens 2006). However, more recently, the use of molecular techniques has helped in the characterization of this taxonomic rank (Johnsen et al. 2006; Phillimore and Owens 2006; Hull et al. 2008b). Although the concept of subspecies is debatable and sometimes subjective, this biological unit may reflect intraspecific diversification and may be indicative of the level of divergence and possibly the adaptive potential (Winker 2010). Subspecies are often studied for management and conservation purposes; however these designations reflect intraspecific genetic variations and may be relevant for understanding the evolutionary history of a species (Johnsen et al. 2006; Phillimore and Owens 2006; Winker 2010).

Study species

To examine (1) the effects of landscape features on gene flow in a complex environment, (2) the relationship between population genetic structure and phenotypic divergence, and (3) the concordance between subspecies designations and intraspecific genetic structuring, I used the muskrat, *Ondatra zibethicus*, as a model organism. The muskrat is a widespread semi-aquatic rodent native of North America (Errington 1963; Willner 1980). Muskrats inhabit a wide range of habitats including streams, lakes and

marshes (Errington 1963; Boutin and Birkenholz1987). They have small home ranges estimated to about 100 m in diameter (Caley 1987; Nadeau et al. 1995; Virgl and Messier 2000) or approximately 582 m in length on average in linear habitats (Ahlers et al. 2010). Muskrats are also territorial during the breeding period (Marinelli and Messier 1993). Because of the biology of this species, such as its small home range and its territoriality, substantial genetic differentiation is expected as is significant isolation by distance. However, other characteristics of the species ecology may promote relatively high rates of gene flow, such as migration of juveniles during the breeding period (Errington 1963), or dispersal due to seasonal changes in water level (Boutin and Birkenholz 1987; Virgl and Messier 1996). Muskrat populations are dependent on the type of water surfaces, water levels and water velocity (Nadeau et al. 1995; Virgl and Messier 1996). Muskrats are also dependent on watersheds for their food supply and shelter (Errington 1963). On land, vegetation cover is an important feature to migration and open habitat such as agricultural land can be considered as a barrier between populations (Virgl and Messier 1996). Like most mammals, the mating system of muskrat has been reported as polygynous (Marinelli and Messier 1993; Marinelli et al. 1997); however Proulx and Gilbert (1983) suggested that muskrats are monogamous. Caley (1987) reported a monogamous mating system with very little polygynous associations. The mating system and social structure of muskrat populations may affect dispersal patterns.

The muskrat displays phenotypic variations across its native range and 16 subspecies have been described mostly based on morphological differences and habits (Errington 1963; Willner 1980). Boyce (1978) detected body size variations across the muskrat North American range. The author found a strong relationship between body size and climatic variables related to primary productivity and attributed this relationship to

natural selection (Boyce 1978). Significant differences in skull morphology have also been identified among muskrat populations in Belgium (Le Boulengé et al. 1996), Finland (Pankakoski and Nurmi 1986) as well as in Canada (Rigby and Threlfall 1982).

Objectives

The purpose of this study was to understand spatial genetic structuring and patterns of gene flow in muskrat populations and relate them to landscape features at different spatial scales. More specifically, the objectives were to 1) develop species specific polymorphic microsatellite loci, 2) examine the relationship between genetic discontinuities and landscape features at a microgeographic scale (approximately 30x30km), 3) examine population genetic structure at a large spatial scale (Ontario) and its relationship with phenotypic divergence, and finally 4) assess the genetic diversity of muskrat populations at a macrogeographic scale (across Canada) and assess its concordance with subspecies designations.

Hypotheses

I hypothesized that at a microgeographic scale, pronounced genetic structure is present due to landscape characteristics including natural and anthropogenic physical barriers. At a large spatial scale, I hypothesized that habitat heterogeneity affects gene flow and lead to substantial population genetic structuring which ultimately leads to phenotypic divergence among these populations. Finally, in small mammal populations, I hypothesized that geographical distances and the presence of physical barriers will lead to population genetic structure and isolation by distance at a macrogeographic scale. I also hypothesized

that subspecies represent genetically distinct units and predicted that the existing subspecies designations should correspond to the population genetic structuring.

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CHAPTER 2. Isolation and characterization of polymorphic microsatellite loci in muskrat, *Ondatra zibethicus*

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Abstract

We describe the isolation and characterization of 12 highly polymorphic microsatellite loci for the muskrat, *Ondatra zibethicus*. Microsatellite markers from three other rodent species were cross-amplified in muskrat and one of them was polymorphic. We observed moderate to high levels of genetic variability in these 13 polymorphic loci (five to 22 alleles per locus) with observed heterozygosity ranging from 0.48 to 0.96. These markers will be useful for further studies on population genetic structure in muskrat and potentially in other rodent species.

Introduction

Anthropogenic disturbances and natural events can result in habitat fragmentation leading to heterogeneous landscapes, and therefore, to the creation of barriers to dispersal. The study of population genetic structure in heterogeneous landscapes is necessary in order to understand how such landscape characteristics affect dispersal and gene flow (Manel et al. 2003). Microsatellite markers have been shown to be useful genetic markers for studying population structure because they are relatively abundant, codominant and they have high mutation rates (Lowe et al. 2004). In order to assess the effect of heterogeneous landscapes on gene flow, we are using the muskrat, *Ondatra zibethicus*, as a model. The muskrat is a widespread mammal in North America that uses the hydrogeographical network as well as terrestrial corridors for dispersal. The study of a semiaquatic species allows consideration of both terrestrial and aquatic features of complex landscapes in the context of population structure and gene flow. Furthermore, the muskrats' wide geographical range will allow us

to investigate the effects of fragmentation on gene flow at macrogeographical and microgeographical scales. Despite its wide geographical range, the muskrat has not been extensively studied and very few population studies based on molecular analyses have been conducted (Marinelli et al. 1997; Zachos et al. 2007). Moreover, microsatellite markers have not been reported as of yet for the muskrat. To address these questions, we have developed polymorphic microsatellite markers for *O. zibethicus*. Here we characterize 12 polymorphic microsatellite markers and test their potential for amplification in five other rodent species (*Microtus pennsylvanicus, Clethrionomys gapperi, Peromyscus maniculatus, Napaeozapus insignis* and *Tamiasciurus hudsonicus*) in order to evaluate their usefulness for further studies on population structure. We also cross-amplified microsatellite markers developed for *M. pennsylvanicus* (Spritzer et al. 2005), *Clethrionomys rufocanus bedfordiae* (Ishibashi et al. 1995) and *P. maniculatus* (Mullen et al. 2006) for their use in muskrat and report the successful amplification of one polymorphic microsatellite locus.

Microsatellite characterization

Microsatellites developed in this study were cloned from an enriched genomic library created using the protocol described in Hamilton et al. (1999). Of 48 clones sequenced, seven resulted in poor or unreadable sequences and eight revealed the repeat region was too close to one end of the clone to allow for the placement of a polymerase chain reaction (PCR) primer. Thirty-five primer pairs were designed from the remaining 33 clones using Primer 3 (version 0.3.0; Rozen & Skaletsky 2000).

Muskrats were collected from trappers across Ontario, Canada, during the 2005–2006 trapping season. Genomic DNA was extracted from muscle tissue using QIAGEN DNeasy procedure. PCR was performed in a 10-μL reaction volume containing 2 μL (> 10 ng) genomic DNA, 10× PCR buffer, 1.5 mm MgCl2, 0.2 mm dNTPs, 0.2 μm of each forward and reverse primers (labeled with HEX or 6-FAM; Table 1), and 0.5 U *Taq* DNA polymerase. PCR amplification was performed using a Mastercycler gradient (Eppendorf). PCR profiles consisted of 2-min initial denaturation at 94 °C, followed by 30 cycles of 1-min denaturation at 94 °C, 30 s at primer-specific annealing temperature (Table 2.1), 30-s extension at 72 °C, and a final extension at 72 °C for 2 min except for locus Oz17 and Oz32 for which the final extension was 45 min. Amplification products were verified on a 1% agarose gel.

Nineteen of the 33 microsatellite loci amplified successfully and polymorphism was assessed by genotyping 10 individuals per locus on a 3730 DNA Analyser (Applied Biosystems) using a GeneScan 600 Liz Size Standard (Applied Biosystems) at MOBIXLab (McMaster University, Hamilton, Canada). Results were analysed using GeneMapper software version 4.0 (Applied Biosystems). Among these 19 loci, 12 loci were polymorphic and genetic variability at each locus was determined by genotyping 30 individuals. Moderate to high levels of genetic variability were observed with a number of alleles per locus ranging from eight to 22 and observed heterozygosity (H_O) ranging from 0.48 to 0.96 (Table 2.1).

Table 2.1. Characterization of 12 microsatellite loci for the muskrat (*O. zibethicus*) and successful amplification of one microsatellite locus from the grey red-backed vole (*C. rufocanus bedfordiae*). Number of alleles (A), expected heterozygosity (H_e), observed heterozygosity (H_o) based on 30 individuals. Significant deviation from Hardy–Weinberg equilibrium (*) are indicated. .

Locus	Genbank accession no.	Primer sequence (5'-3')	Repeat motif	T_a (°C)	size range (bp)	A	H_e	H_o
Oz06	EU999728	F: GGACAACAGAGAGGGAAGGA R: CTCATATTGTAAGAAGCCTGCTG†	$(AC)_{20}$	65	135-181	15	0.93	0.93
Oz08	EU487259	F: CCTATGGGACTGACGGCTAA† R: AGTTTGGGACTCTGCCTTGA	$(CA)_5AACACA(TC)_5TG(TC)_7(AC)_8$	63	231-251	8	0.82	0.79
Oz16	EU999729	F: TGACTGCACTGTTCCACACA R: AAGCATCTCTGCTGGGTCAT‡	$(CA)_{22}$	67	289-335	18	0.93	0.96
Oz17	EU487260	F: GCAAGGCACCTAAGTGTGTG R: TTGGGTCTTCACTGGGTAGC‡	$(GT)_{18}\ldots(AG)_{22}$	63	174-228	22	0.96	0.89*
Oz22	EU487262	F: GTCTGTCTCGCGCTCTCTCT‡ R: CGCTCCCCAAACCTGTACTA	$(CT)_{23}(CA)_{12}$	63	207-241	15	0.90	0.48*
Oz27	EU999730	F: GCTGAAATGAAACTGGCTAA R: TCTGAACTGGTGTGGGATTG†	$(AC)_{18}$	64	197-217	10	0.89	0.82
Oz30	EU487263	F: GCTTCGGTGACAATGGAAAT R: TTCGTGGCTGAATACCCAGT†	$(GA)_{31}AACA(GA)_{12}$	63	212-254	19	0.95	0.74

Locus	Genbank accession no.	Primer sequence (5'-3')	Repeat motif	T_a (ºC)	size range (bp)	A	H_e	H_o
Oz32	EU999731	F: TTGACTTTTTCCAACATTCAGAG‡ R: TTGCAATTCTGTGGCTAGGA	$(GA)_{18}TA(GA)_{12}$	65	172-206	13	0.91	0.96
Oz34	EU999732	F: ACCTTCCATTCTTAAATAGC† R: GATTCTCTCTCTTTCACTCAT	$(GT)_5AT(GT)_5\ldots(GA)_7\ldots(GT)_{13}$	60	216-232	9	0.83	0.79
Oz41	EU487264	F: ATGACATTGACCCAGGGAAG† R: GACGGTGCAGATTTGGTTCT	$(CA)_{40}$	63	180-248	21	0.95	0.83
Oz43	EU487265	F: AGAAGGGAGTGAGCACCTGA‡ R: CTAGCCCCCATAGGCATGTA	$(TG)_{16}$	64	230-278	15	0.91	0.83
Oz44	EU999733	F: TCCAGAGAGGTTACCGAAATG† R: CCCTTCAGGACCAGTGTCAT	$(CA)_{19}$	63	215-257	11	0.68	0.50
MSCRB5[(1)]	-	F: GGTTGGTGTTTGCATTTAGG‡ R: CTCCTGGTAATTTTCATCTTACC	CA-, ATAC-, ATGT-	48	186-202	5	0.68	0.50

Primers labeled with: †6-FAM; ‡HEX

[(1)] Ishibashi et al. (1995)

The 12 polymorphic loci developed for *O. zibethicus* showed some level of consistent cross-amplification in five other rodent species (Table 2.2). The PCR protocols followed those used for muskrat with the exception of the annealing temperature, which is indicated in Table 2. The success of these cross-specific amplifications was verified using gel electrophoresis; however, the amplified products were not genotyped on a sequencer. These loci may have some potential for further studies on population structure in these species with the exclusion of *T. hudsonicus*.

Eight markers from other rodent species were also tested on *O. zibethicus* including four loci developed for *M. pennsylvanicus* (AV13, AV14, AV15 and MSMM2; Spritzer et al. 2005), two loci developed for *C. rufocanus* (MSCRB2 and MSCRB5; Ishibashi et al. 1995) that have been successfully used for *C. gapperi* in other studies (Mech & Hallett 2001; Reese et al. 2001), and two loci developed for *P. maniculatus* (Bw4-28 and Bw4-249, Mullen et al. 2006). Three of these eight loci showed amplification in *O. zibethicus*; one was polymorphic (MSCRB5; Table 2.1), one was monomorphic (MSCRB2; *T*a = 54 °C) and one exhibited inconsistent amplification and was therefore removed from further analysis (AV13; *T*a = 59 °C).

For the 13 polymorphic loci amplifying in *O. zibethicus*, we tested for departure from Hardy–Weinberg equilibrium (HWE) using the software GenePop version 4.0.7 (Rousset 2008). Two loci exhibited significant departure from HWE (Oz17 and Oz22) even after sequential Bonferroni correction ($P = 0.0035$ and $P < 0.0001$ respectively). By using only 10 individuals from one region (Sudbury), only one locus (Oz22) deviated from HWE after sequential Bonferroni correction ($P < 0.0001$). The deviation from HWE may be explained by the presence of null alleles.

Table 2.2. Cross-species amplification of 12 microsatellite loci developed for the muskrat, based on five individuals per species. Annealing temperature (ºC) is showing in parentheses.

	Microtus pennsylvanicus	*Clethrionomys gapperi*	*Peromyscus maniculatus*	*Napaeozapus insignis*	*Tamiasciurus hudsonicus*
Oz06	-	-	+ (61)	-	-
Oz08	-	-	-	-	-
Oz16	-	+ (59)	+ (60)	+ (58)	-
Oz17	-	+ (64)	-	-	-
Oz22	-	-	+ (56.5)	+ (59)	-
Oz27	+ (60)	+ (64)	+ (55)	-	-
Oz30	+ (62.5)	-	-	-	-
Oz32	-	+ (64)	-	-	-
Oz34	-	-	-	-	-
Oz41	+ (63)	+ (63)	+ (60)	-	-
Oz43	+ (62)	+ (61)	-	-	-
Oz44	+ (61)	-	-	-	-

-, no amplification or inconsistent product; +, amplification product.

The presence of null alleles was tested using Micro-Checker version 2.2.3 (van Oosterhout et al. 2004) with a confidence interval of 99% and 5000 randomizations. One of the loci showing deviation from HWE (Oz22) showed significant excess of homozygotes ($P < 0.001$) which could indicate the presence of null alleles (frequency of null alleles $r = 0.296$). Linkage disequilibrium was tested on all loci using fstat version 2.9.3.2 (Goudet 2002) and results suggested that loci Oz08 and Oz16 as well as loci Oz27 and Oz32 displayed significant linkage disequilibrium after Bonferroni correction ($P = 0.00064$ for both pairs). These new microsatellite loci will be useful to estimate population genetic structure in *O. zibethicus* and may potentially be useful for population studies in other rodent species.

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CHAPTER 3. Effects of structural connectivity on fine scale population genetic structure of muskrat, *Ondatra zibethicus*

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Abstract

In heterogeneous landscapes, physical barriers and loss of structural connectivity have been shown to reduce gene flow and therefore lead to population structuring. In this study, we assessed the influence of landscape features on population genetic structure and gene flow of a semi-aquatic species, the muskrat. A total of 97 muskrats were sampled from three watersheds near Sudbury, Ontario, Canada. We estimated population genetic structure using 11 microsatellite loci and identified a single genetic cluster. No genetic differences were found among the watersheds as a result of high levels of gene flow. At finer-scales we assessed the correlation between individual pairwise genetic distances and Euclidean distance as well as different models of least cost path (LCP). We used a range of cost values for the landscape types in order to build our LCP models. We found a positive relationship between genetic distance and least cost distance when we considered roads as corridors for movements. Open landscapes and urban areas seemed to restrict but not prevent gene flow within the study area. Our study underlines the high dispersal ability of generalist species in their use of landscape and highlights how landscape features often considered barriers to animal movements are corridors for other species.

Introduction

Contemporary population structure can be affected by ecological barriers and decreased structural connectivity between optimal habitat patches (Coulon et al. 2004; Storfer et al. 2007). Recent events including anthropogenic activities and urban development may increase the loss of optimal habitat and decrease connectivity between populations (Cushman 2006; Riley et al. 2006) ultimately resulting in geographical isolation (Trizio et

al. 2005; Vandergast et al. 2007). However, Crispo et al. (2011) underlined the positive effects of human activities on gene flow for several species. Anthropogenic features such as roads have usually been shown to negatively affect dispersal and gene flow (Holderegger and Di Giulio 2010). However, some studies have reported positive effects of roads acting as corridors to animal movement (Holderegger and Di Giulio 2010; Crispo et al. 2011). This emphasizes the importance of understanding patterns of gene flow, particularly in complex environments. Features that are a barrier to animal movements in some species may facilitate gene flow in others. The restriction to a specific environment, such as the aquatic environment, increases the effects of barriers on gene flow and therefore can lead to substantial genetic structure (Mullen et al. 2010; Mikulíček and Pišút 2012). The degree of dependence on the aquatic environment varies among semi-aquatic species and the terrestrial connectivity between these aquatic habitats is critical for dispersal movements and hence gene flow (Carranza et al. 2012).

Assessing the relationship between gene flow and landscape can be performed by developing a cost surface. Methods used to characterize landscape costs in order to measure resistance surfaces are developing rapidly (Sawyer et al. 2011). Whether using a least cost path (LCP) model or circuit theory (McRae et al. 2008), cost values have to be attributed to the different landscape features. The choice of these cost surfaces is species-specific and usually subjective as it is often based on expert opinion (Rayfield et al. 2010; Koen et al. 2012). The assignment of cost surfaces remains one of the challenges of the assessment of functional connectivity, especially in light of the fact that the location of the LCP is sensitive to the relative cost values (Rayfield et al. 2010), and the accumulated cost of the LCP (i.e., the cost distance) increases linearly with increasing relative cost weights (Koen et al. 2012).

Other factors that determine the effect of landscape on population genetic structure are closely linked to the dispersal ability and movement behavior of a species (Clark et al. 2008; Cushman and Lewis 2010). High vagility during natal and/or breeding dispersal may enhance gene flow, whereas strong philopatry may decrease it (Temple et al. 2006; Ortego et al. 2011), thus increasing genetic differentiation and population genetic structure. However, even species with relatively high dispersal ability may present population genetic structure at fine spatial scales due to the influence of landscape structure (Booth et al. 2009; Neaves et al. 2009). Dispersal behaviours differ among species and vary depending on social structure and mating system (Lawson Handley and Perrin 2007). Differences between the sexes in dispersal have also been shown to affect the genetic structure of populations (Nussey et al. 2005; Chambers and Garant 2011).

We analysed gene flow in muskrat (*Ondatra zibethicus*) and assessed the effect of landscape features on population genetic structure. This semi-aquatic rodent is widespread across North America and uses a wide range of freshwater habitats such as streams, marshes and lakes (Boutin and Birkenholz 1987). Muskrats are dependent on the hydrographic network for shelter, food resources and reproduction (Boutin and Birkenholz 1987; Ahlers et al. 2010) but they also have the capacity to use terrestrial pathways during dispersal (Errington 1963). Very little is known about the movement abilities of muskrat over different types of terrain and what types of features are considered barriers to movements. Muskrats have small home ranges: approximately 100 m in diameter (Boutin and Birkenholz 1987; Caley 1987) or 582 m in length in linear habitats (Ahlers et al. 2010). They also have limited natal dispersal (less than 100 m on average) (Caley 1987) as well as limited adult dispersal (30 m to 5 km on average; Errington 1963) during the breeding season or when disturbances occur in the environment such as drought or freezing (Boutin

and Birkenholz 1987). Dispersal has been reported as male-biased in muskrat populations (Caley 1987) but it may vary depending on the social structure and mating system (Lawson Handley and Perrin 2007).

We examined the effect of landscape features on the population genetic structure of semi-aquatic muskrat using varied cost surfaces to characterize the landscape in order to take into account the path's sensitivity to the cost values and to assess their respective effect on the relationship between genetic distances and least cost path. Semi-aquatic species may display different patterns of population genetic structure than strictly terrestrial or aquatic species, and this can have important consequences for species conservation and management in fragmented landscapes. We hypothesized that at a fine spatial scale, fragmentation limits dispersal of muskrats. We predicted that muskrat should exhibit population genetic structure because of the landscape heterogeneity (aquatic and terrestrial) and the presence of anthropogenic features (e.g. roads). More specifically, because of the biology of the species, we predicted that muskrats will show population genetic structure which will reflect the watershed structure and that genetic differentiation should be greater between than within watersheds.

Methods

Sample collection

Muskrats (N=97) were live-trapped in three watersheds located in Sudbury District, Ontario, Canada during May, June and July of 2008 (Figure 3.1).

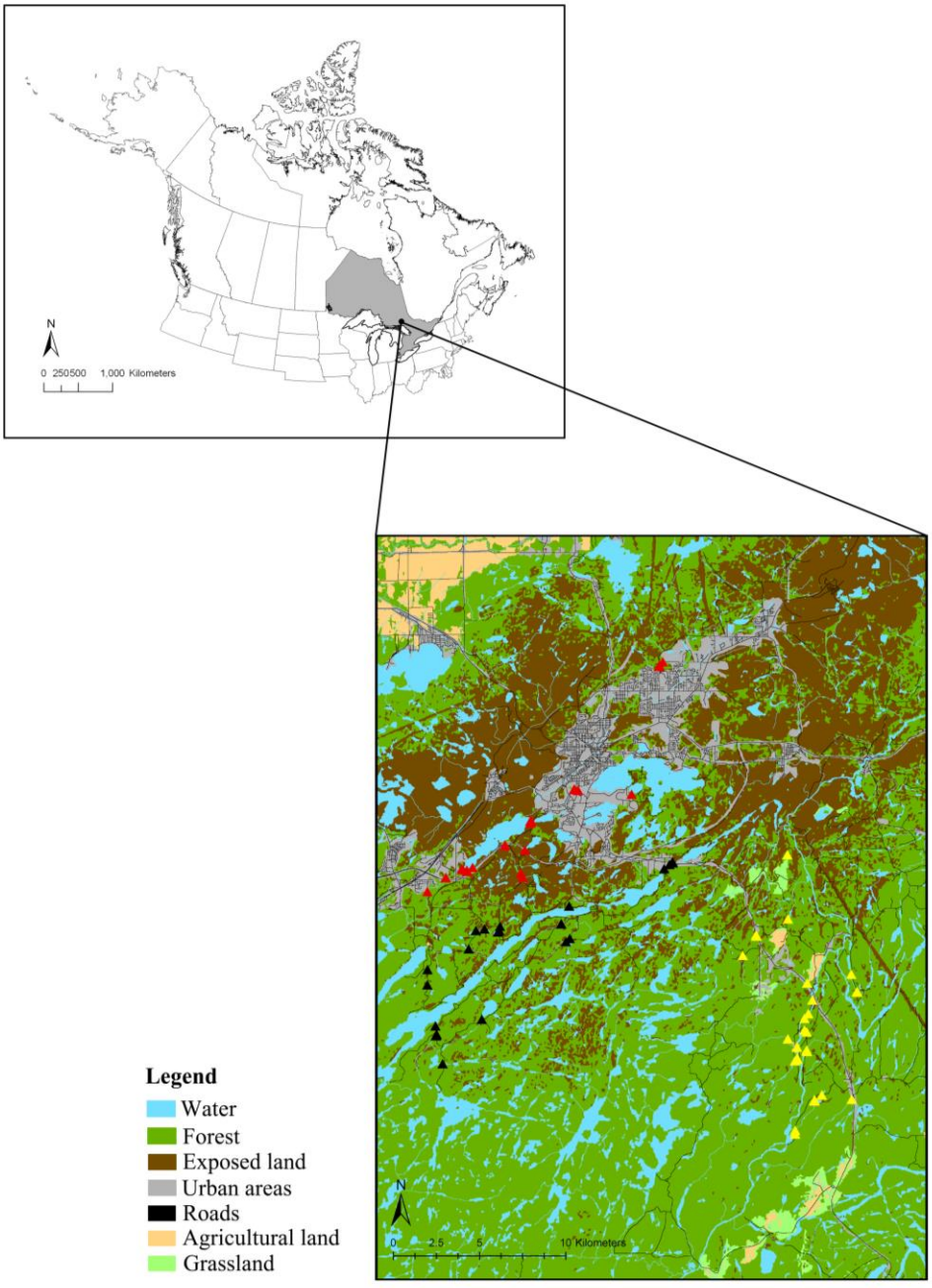

Figure 3.1. Study area showing the locations of muskrats from the three watersheds: Upper and Lower Junction Creek (▲), Panache (▲), East Wanapitei (▲) with the landcover types.

Animal trapping and handling was done according to the procedures of the Animal Care Committee of Laurentian University (protocol #2007-04-01) and a Wildlife Scientific Collector's Authorization issued by the Ontario Ministry of Natural Resources (#1039126). Site selection along each watershed was based on accessibility and presence of suitable muskrat habitat. The geographical coordinates of each trap location were recorded using a GPS (Garmin GPSMAP 60Cx; accuracy <10 m). Each individual was marked with an ear tag, weighed, and tissue samples (ear clip) were collected for genetic analysis. We collected 32 samples from the Upper and Lower Junction Creek watershed (Jc), 31 samples from the Panache watershed (Pa) and 34 samples from the East Wanapitei watershed (Wa) (Figure 3.1).

Sex determination was performed following the methods described in Ermakov et al. (2006) using the Smc8D and Smc9R primers. Of the 97 individuals sampled, 64 were identified as males and 33 were females. Age was estimated as adult (> 1 year old) or juvenile (< 1 year old). Because the animals were live trapped and not immobilized, we estimated age using total body mass. Muskrat adult average mass ranges from 900 g to 1400 g (Boutin and Birkenholz 1987). In our study, to be conservative, we considered all individuals less than 1000 g to be juveniles (n=20). The total number of adults was 77, of which 54 were males and 23 were females. Analyses of genetic structure with or without juveniles had no consequences for the resulting output (data not shown), therefore we maintained all samples in our analyses.

Genetic analyses

DNA was extracted using QIAGEN DNeasy tissue kits. All individuals were genotyped at 11 microsatellite loci (Oz06, Oz08, Oz16, Oz22, Oz27, Oz32, Oz34, Oz41, Oz43, Oz44,

MSCRB5) following Laurence et al. (2009). PCR products were run on an ABI 3730 sequencer and fragment size was determined using Peak Scanner v1.0 (Applied Biosystems, Foster City, CA, USA).

MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004) was used to test for genotyping errors and for the presence of null alleles, with a confidence interval of 95% and 5000 randomizations. We tested for deviations from Hardy-Weinberg equilibrium (HWE) using GENEPOP version 4.0.7 (Rousset 2008) and linkage disequilibrium (LD) using FSTAT v.2.9.3 (Goudet 2002). To control for multiple tests, sequential Bonferroni corrections (alpha = 0.05) were used to adjust the level of significance of HWE and LD (Rice 1989).

Genetic diversity was estimated using allelic richness (A), observed (Ho) and expected (He) heterozygosity. Allelic richness was calculated using rarefaction in HP-RARE to control for differences in samples sizes (Kalinowski 2005). Differences in genetic diversity among watersheds were tested using the nonparametric Kruskal-Wallis test (Statistica version 6). Pairwise genetic distance between individuals was determined using the proportion of shared alleles (D_{ps}) and Rousset's a (a_r) which were calculated using Microsatellite Analyzer (MSA) (Dieringer and Schlötterer 2003) and SPAGeDi 1.3 (Hardy and Vekemans 2002), respectively.

Effect of sex on dispersal

Sex-biased dispersal was investigated using five different tests across loci: F_{IS}, F_{ST}, relatedness, mean assignment index (mAIc), and the variance of these assignment indices (vAIc), implemented in FSTAT v.2.9.3 (Goudet et al. 2002). The dispersing sex will show

a higher and positive F_{IS}, lower F_{ST} value, relatedness and mAIc and a higher vAIc than the philopatric sex (Goudet et al. 2002).

We also performed spatial autocorrelation analyses of cumulative distance classes among all trapping locations using the program GENALEX 6.5. (Peakall and Smouse 2012) in order to measure the extent of spatial genetic structure and relatedness for each sex. The analysis was performed for all individuals combined as well as for the sexes separately. We used variable distance classes as the individuals were unevenly distributed across the distances. The distance classes were chosen to maximise the number of pairwise comparisons. However, because of the small sample size of adult females, we were not able to reach 100 pairs as recommended by Hardy and Vekemans (2002). For each distance class, a correlation coefficient was calculated using pair-wise genetic distance and geographic distances as implemented in GENALEX 6.5. (Peakall and Smouse 2012). The 95% confidence intervals of the null hypothesis of random distribution were determined using 999 permutations and the 95% confidence intervals for the autocorrelation coefficient were estimated using 1000 bootstraps. The significance of the difference between the sexes for each distance class was also tested using the test for heterogeneity. These sex-biased dispersal analyses were performed on adults only, as they are the individuals of reproductive age and had already dispersed at the time of sampling.

Population genetic structure

We used several approaches to estimate population genetic structure (Ball et al. 2010; François and Durand 2010). First, we used two individual-based clustering approaches without *a priori* defined populations: STRUCTURE v. 2.3.1 (Pritchard et al. 2000), a non-spatial Bayesian clustering method, and TESS v.2.3 (Durand et al. 2009) which includes

individual spatial information. We ran STRUCTURE with five independent runs per K, with K ranging from one to ten, assuming admixture and correlated alleles. Each run was conducted with a burn-in of 500,000 followed by 500,000 iterations. The most probable K was assessed from the posterior probabilities for each value of K (Pritchard et al. 2000) in addition to the ΔK (Evanno et al. 2005) as well as from the probability of membership of each individual (q) averaged over the five runs. We ran TESS v.2.3 (Durand et al. 2009) under the assumption of admixture. We ran 50,000 MCMC iterations with 20,000 burn-in for 100 runs, with K=2 to K=10. The most likely K was chosen from the deviance information criterion (DIC) values. Second, we also performed a Principal Component Analysis (PCA), a multivariate method adapted to genetic markers, implemented in the Adegenet package in R (Jombart 2008). Pairwise Fst values were calculated for the three watersheds using permutations to test for significance as implemented in FSTAT v.2.9.3 (Goudet et al. 2002).

Least cost path analysis

Landscape data for the Sudbury district were obtained from the National Hydro Network GeoBase (2004) for the water bodies data, Statistics Canada (2006) for the road network and Land Cover Circa GeoBase (2000) for the land cover. All of the landscape characteristics of our study area were aggregated into three or four types of landscape cover that could potentially impact muskrat movement (positively or negatively). We reclassified the landscape types into categories that corresponded to low, medium and high cost for muskrat movements, based on previous studies of muskrat spatial ecology (Errington 1963; Virgl and Messier 1996; Ahlers et al. 2010). Because we did not know the effect of roads on muskrat movements, we built two raster maps. The first one consisted of three

categories of landscape: water, forest and “open landscape and human activity”. The category “open landscape and human activity” included grassland, exposed land (i.e. rock outcrop, barren land), agricultural land, roads and urban areas (i.e. residential, commercial, industrial). The second raster map consisted of four categories: water, roads, forest and “open landscape and human activity” (which combined grassland, exposed land, agricultural land and urban areas). We also included dams and waterfalls as impermeable barriers. These land cover types were mapped on raster maps with a cell size of 20 m by 20 m.

We allocated resistance values to each cell in order to calculate least cost paths (LCP). However, resistance values are often chosen arbitrarily and the range of these values are also variable (Sawyer et al. 2011). In order to take into account the effect of the values chosen, we built several models with different cost schemes (see Table 3 for examples of models) (Desrochers et al. 2010; Sawyer et al. 2011). Pairwise LCP distances in meters were calculated for each cost model using Pathmatrix 1.1 (Ray 2005), an extension of the geographical information system software ARCVIEW 3.X (Environmental Science Research Institute, Redlands, USA).

We compared pairwise genetic distances to Euclidean distances and the different effective distances (33 LCP models) using partial Mantel tests with 10,000 permutations, which were calculated using the package ecodist version 1.2.7 (Goslee and Urban 2007) implemented in R 2.13.0 (R Development Core Team 2011). We used partial Mantel tests in order to control for the effects of Euclidean distance on the relationships between the genetic distances and the LCP models. P-values were adjusted for multiple tests using false discovery rates (FDR) (Pike 2011).

Results

Genetic structure

No evidence of genotyping errors was found and two loci (Oz08 and Oz22) were suspected to show presence of null alleles (estimated % of null alleles: 12.1% and 19.1% respectively). We did not detect LD after Bonferroni corrections and two out of 11 loci were not in HWE after sequential Bonferroni corrections (Oz08 $p<0.001$ and Oz22 $p<0.0001$). These two loci were removed from further analyses so all the results presented were obtained using nine microsatellite loci.

Genetic diversity was not significantly different among the three watersheds (Kruskal-Wallis $p>0.05$) and was highly diverse in all three regions (Table 3.1). Overall mean number of alleles per locus (A) was 15.8 (±5.74) with A ranging from 11.3 to 12.1 (Table 3.1). Observed and expected heterozygosities for all samples were 0.81 (±0.11) and 0.83 (±0.09) respectively (Table 3.1).

We did not detect any significant sex-biased dispersal (Table 3.2). However, although not statistically significant, four of the five tests used to examine sex bias in dispersal showed a tendency toward male-biased dispersal with a higher F_{IS}, lower F_{ST} and lower relatedness (Table 3.2). The mAIc was higher and positive for females (0.581) and negative for males (-0.252), but again not statistically significant. The variance of AIc is expected to be higher in the dispersing sex; however, we found that vAIc was higher in females but not significant, indicating a tendency toward female-biased dispersal (Table 3.2).

Table 3.1. Genetic diversity of muskrat (*Ondatra zibethicus*) in the three watersheds in the Sudbury District, Ontario. Number of individuals (N), allelic richness (A), expected heterozygosity (He), observed heterozygosity (Ho) are indicated with standard deviation in brackets.

Watershed	N	A	He	Ho
Junction creek	32	11.8(4.00)	0.81(0.11)	0.81(0.14)
Panache	31	12.1(4.12)	0.82(0.08)	0.78(0.08)
East Wanapitei	34	11.3(3.73)	0.82(0.10)	0.85(0.13)
Total	97	15.8(5.74)	0.83(0.09)	0.81(0.11)

Table 3.2. Results of sex-biased dispersal tests in adult muskrats (*Ondatra zibethicus*). Significance values were calculated using 5000 permutations

	F_{IS}	F_{ST}	Relatedness	Assignment Indices	
				Mean	Variance
Females (n=23)	0.041	0.019	0.035	0.581	17.330
Males (n=54)	0.074	0.012	0.022	-0.252	9.239
P values	0.269	0.380	0.370	0.204	0.988
Overall (n=77)	0.068	0.010	0.019	-	-

The spatial autocorrelation analyses of cumulative distance classes showed similar patterns of genetic structure in both sexes (Figure 3.2B). Both sexes had correlation coefficients not significant from random for any of the distance classes. Females displayed a positive r-value within the 0 to 3 km distance class (r=0.033), however it was not significant. The test for heterogeneity did not detect a difference in spatial genetic structure patterns between sexes ($0.200 \leq p \geq 0.991$) indicating homogeneity between the spatial correlograms.

Both type of analyses indicated a lack of sex-biased dispersal. However, the tests from Goudet et al. (2002) showed an absence of sex biased dispersal with a tendency toward male biased dispersal and the spatial autocorrelation analysis indicated a tendency toward female philopatry under 2.5 km. Consequently, the genetic structure analyses were performed for all samples as well as for the sexes separated. We did not detect any differences in the results when separating the sexes and therefore all the results presented henceforth include male and female samples combined. The spatial autocorrelation analysis of cumulative distance classes indicated that individuals *rc* (males and females pooled) were not significantly different from random for all the distance classes (Figure 3.2A).

A single cluster (K=1) was suggested by STRUCTURE. Although the highest LnP(K) was detected at K=5 (average LnP(K) =-4025), suggesting the presence of five clusters, the proportion of individuals ancestry (q) was low (0.377 ±0.178). These results indicate that K=5 is not the true K and the number of genetic clusters is one. Similar results were obtained using the spatial Bayesian clustering method TESS.

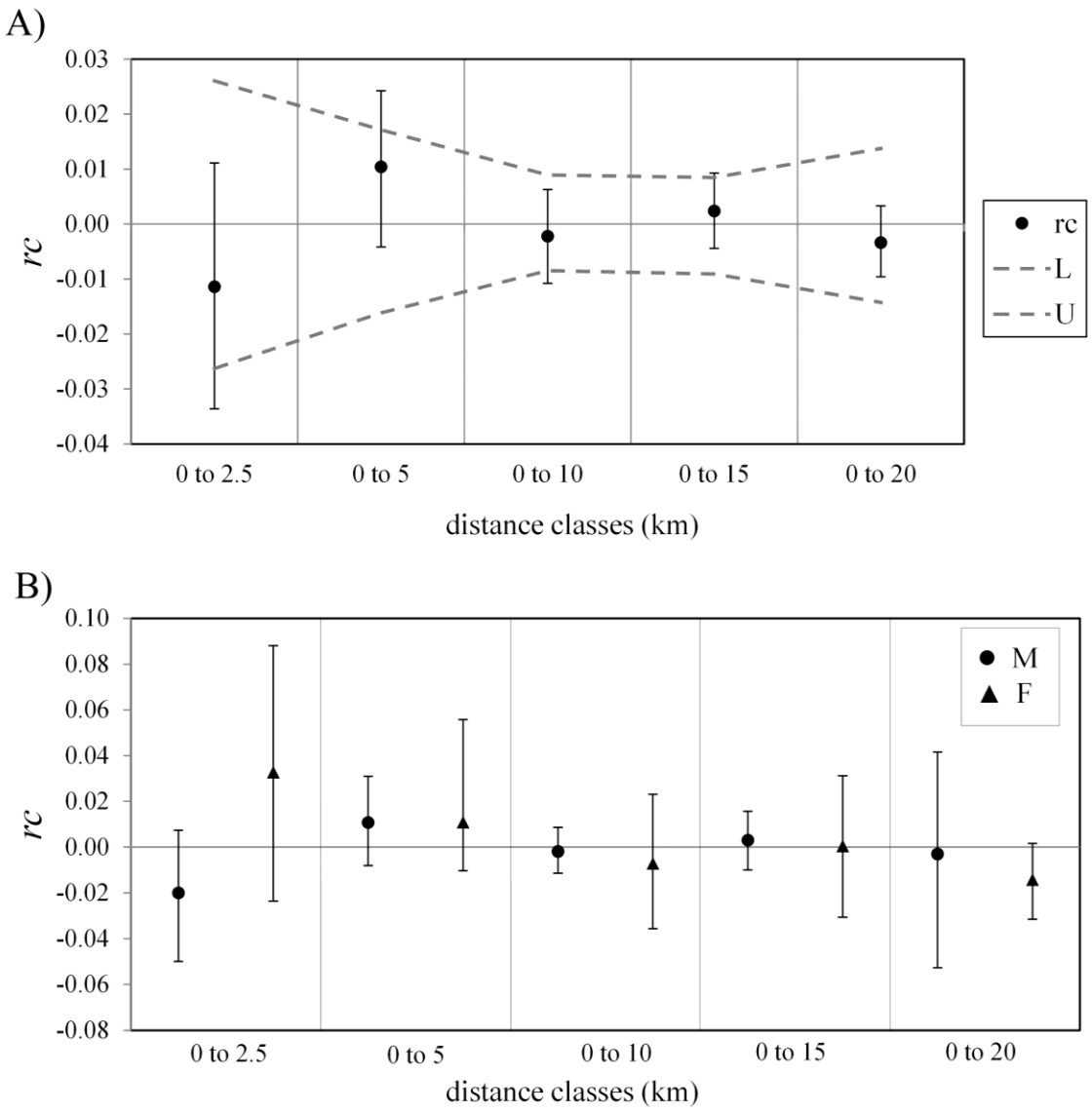

Figure 3.2. Spatial autocorrelograms of the cumulative distance classes. Correlation coefficients are presented for all individuals (A) and males and females (B). The 95% confidence error bars and the permuted 95% confidence intervals (dashed lines) for the null hypothesis of random distribution are presented.

Comparable results indicating one cluster were also obtained using the PCA (Figure 3.3) with 8.2% of the variance explained by the first axis and 6.3% of the variance explained by the second axis, providing further evidence that muskrats from the three individual watersheds were not genetically distinct. Pairwise F_{st} values were significantly different from zero between East Wanapitei and Junction creek ($F_{st} = 0.0174$, $p \leq 0.05$) and between East Wanapitei and Panache ($F_{st} = 0.0164$, $p \leq 0.05$) but not between Junction creek and Panache ($F_{st} = 0.0001$).

Least cost path analysis

The full Mantel tests performed between the genetic distances (a_r and D_{ps}) and the Euclidean distance were significant ($p = 0.0002$ and $p = 0.0001$ respectively), but showed weak Mantel r (0.108 and 0.154 respectively; Table 3.3). After partialling out the Euclidean distance from the LCP models, the only significant relationships after adjusting the p-values for multiple tests ($0.014 < p < 0.012$) were found between D_{ps} and LCP models that considered the roads as facilitator models for muskrat movements (Table 3.3).

Discussion

One panmictic population was detected in our study area and no evidence of genetic differentiation within or among the three watersheds was observed. The population had a high genetic diversity. These results were consistent among the various approaches used to estimate population genetic structure at a fine spatial scale. This continuous distribution of muskrat with no genetic differentiation between watersheds suggests substantial gene flow throughout the study area.

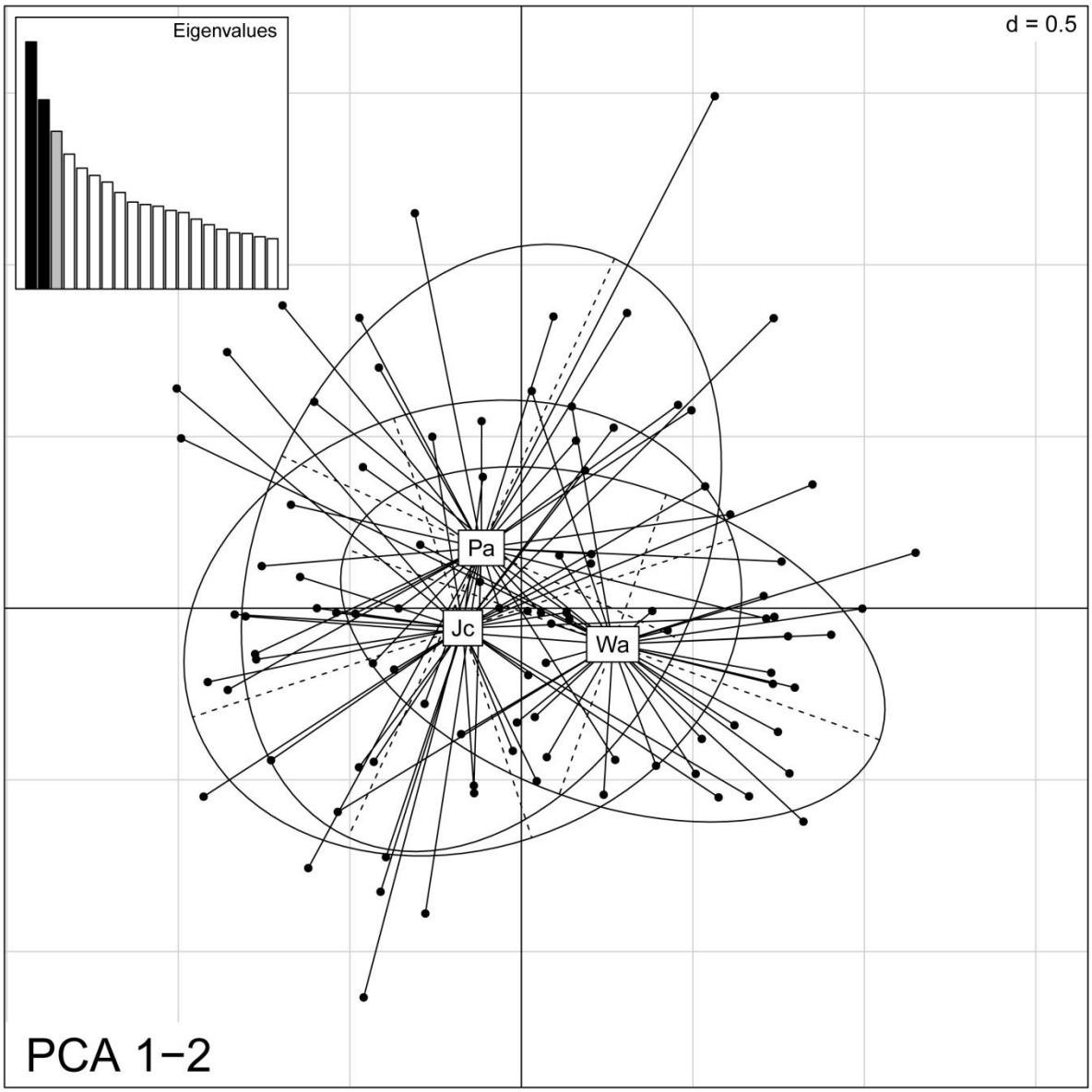

Figure 3.3. PCA of the first two principal components for the three watersheds: Upper and Lower Junction Creek (Jc), Panache (Pa) and East Wanapitei (Wa).

Table 3.3. Results of partial Mantel tests between the genetic distances (a_r and D_{ps}) and the geographic distances in meters: Euclidean distance (first row) and the different LCP models (4 categories of landscape). All partial Mantel tests are partialling out the Euclidean distance. 95% confidence intervals (CI) are indicated in parentheses.

Resistance to movement				a_r		D_{ps}	
Water	Roads	Forest	Open landscape + human activity	Partial Mantel's r (CI)	P	Partial Mantel's r	p
1	1	1	1	0.108 (0.080 to 0.134)	0.0002	0.154 (0.129 to 0.176)	0.0001
1	1	2	4	-0.026 (-0.054 to 0.005)	0.764	-0.014 (-0.040 to 0.007)	0.681
1	1	5	10	0.018 (-0.013 to 0.049)	0.336	0.053 (0.030 to 0.077)	0.063
1	1	5	50	0.011 (-0.018 to 0.041)	0.390	0.050 (0.025 to 0.073)	0.067
1	1	10	20	0.026 (-0.009 to 0.060)	0.251	0.058 (0.033 to 0.081)	0.037
1	1	10	100	0.029 (-0.0001 to 0.063)	0.218	0.062 (0.037 to 0.084)	0.029
1	1	25	50	0.024 (-0.003 to 0.053)	0.271	0.060 (0.035 to 0.083)	0.033
1	1	50	100	0.035 (-0.0002 to 0.064)	0.190	0.077 (0.052 to 0.102)	0.012*
1	1	50	500	0.035 (0.006 to 0.064)	0.189	0.077 (0.054 to 0.100)	0.012*
1	1	100	200	0.031 (-0.001 to 0.057)	0.223	0.078 (0.055 to 0.101)	0.012*

1	1	100	1000	0.031 (-0.0002 to 0.060)	0.231	0.078 (0.054 to 0.104)	0.011*
1	1	250	500	0.031 (0.002 to 0.059)	0.223	0.078 (0.051 to 0.104)	0.012*
1	1	500	1000	0.030 (0.002 to 0.059)	0.232	0.078 (0.053 to 0.103)	0.014*
1	10	10	20	-0.002 (-0.024 to 0.023)	0.527	0.014 (-0.007 to 0.035)	0.334
1	10	10	100	-0.012 (-0.038 to 0.017)	0.620	-0.007 (-0.030 to 0.014)	0.595
1	10	25	50	-0.020 (-0.043 to 0.005)	0.692	-0.013 (-0.034 to 0.012)	0.657
1	10	50	100	-0.043 (-0.071 to -0.014)	0.842	-0.021 (-0.045 to 0.005)	0.721
1	10	100	200	-0.043 (-0.068 to -0.011)	0.835	-0.030 (-0.053 to -0.001)	0.784
1	10	100	1000	-0.043 (-0.068 to -0.011)	0.846	-0.030 (-0.051 to -0.004)	0.793
1	10	250	500	-0.027 (-0.052 to -0.002)	0.745	-0.007 (-0.029 to 0.018)	0.586
1	10	500	1000	-0.026 (-0.048 to -0.001)	0.740	-0.009 (-0.032 to 0.019)	0.599

* significant result using false discovery rates adjusted p-values

The individual based approaches (STRUCTURE, TESS and PCA) did not demonstrate genetic structure at the fine geographical scale. Underlying patterns of isolation by distance can make it difficult to interpret the results from Bayesian clustering methods (Pritchard et al. 2000; Frantz et al. 2009) and these results should be taken with caution. Nevertheless, we also detected one population using the ordination method (PCA). Pairwise F_{st} values indicated significant differentiation between East Wanapitei and the other watersheds. However, using a priori defined populations may lead to significant F_{st} values particularly when an isolation by distance pattern is present (Gauffre et al. 2008, Frantz et al. 2010; Wasserman et al. 2010); which is most likely the case in our study. The spatial autocorrelation analysis of cumulative distance classes did not detect spatial genetic structuring in relation to distance at this fine spatial scale. Although there is limited information regarding the dispersal capacities of muskrats, Errington (1963) reported limited dispersal distances ranging from 30 m to 5 km with the majority of individuals dispersing to a maximum of 100 m. However, muskrats have the ability to travel over long distances particularly in response to extreme conditions such as drought or high population density, with individuals dispersing to up to 34 km (Errington 1963). Artimo (1960) reported dispersal distances of 4 to 120 km per year in Finland with the majority of individuals dispersing within the distance category of 10-20 km.

We did not detect spatial genetic structuring for both sexes suggesting a lack of sex-biased dispersal at this spatial scale. Dispersal in mammals is usually biased toward males (reviewed by Lawson Handley and Perrin 2007) however, we did not detect clear evidence of sex-biased dispersal in this study as suggested by the spatial autocorrelation analysis that showed similar patterns in both sexes. Although the lack of sex-biased dispersal in mammals has rarely been observed, it has been reported in several mammals such as the

European roe deer (*Capreolus capreolus*) (Bonnot et al. 2010) and the southern water vole (*Arvicola sapidus*) (Centeno-Cuadros et al. 2011). Social and mating systems influence sex bias in mammal dispersal (Lawson Handley and Perrin 2007). Polygynous species often display male-biased dispersal whereas monogamous species display female-biased dispersal, however this rule does not apply for several species (Lawson Handley and Perrin 2007). Muskrats have been reported to be polygynous in some regions and monogamous in others (Marinelli et al. 1997) suggesting that the mating system alone cannot explain the absence of bias in dispersal in this species. Patterns of breeding dispersal and natal dispersal may also influence dispersal and explain the lack of bias in some species (Coulon et al. 2006). Further study on the social structure of muskrat population is necessary in order to better understand dispersal patterns. We did observe a tendency of female philopatry up to 2.5 km, however it was not significant. One of the limitations of these results is the small female sample size, which reduces the power of the tests (Lawson Handley and Perrin 2007).

Contrary to our hypothesis, our results showed that population genetic structure was not influenced by the landscape structure at a fine geographical scale. However, genetic distances among individuals were partly explained by landscape features. We detected only one population in our study area and no barriers were identified which seems to support the idea that no landscape features seemed to prevent gene flow. However, when comparing the different models, models with high cost for "open landscape and urban areas" and "forest" were the models with the highest significant partial mantel's r, which seems to indicate that they do prevent, to some extent, gene flow. Open landscape and urban areas and to some extent forest seemed to restrict but not prevent gene flow in muskrat. Muskrat movements are influenced by the landscape, but gene flow was not prevented by the

landscape characteristics in our study and no landscape barrier was identified. Although Ahlers et al. (2010) found that muskrat home ranges were linear and restricted to river banks; we did not find that gene flow was limited to the watershed. This linear use of habitat may be explained by the substantial presence of agricultural land in their study sites preventing muskrat movements outside of stream networks. Our study area did not contain a high percentage of agricultural land (approximately 1%), thus allowing individuals to use non-agricultural terrestrial corridors. Our results suggest a limited effect from landscape features and presence of isolation by distance. Gauffre et al. (2008) did not detect any barrier to gene flow in common vole (*Microtus arvalis*) populations. The authors explained this isolation by distance pattern and the lack of effect of landscape fragmentation on gene flow by the high effective population size of this species and the barrier being too recent to affect such a large population.

Stream connectivity has been shown to influence patterns of genetic structure in amphibian species (Mullen et al. 2010). In semi-aquatic mammals this relationship has rarely been demonstrated because of their ability to use terrestrial corridors (Vignieri 2005; Zalewski et al. 2009). Similarly, we did not find that muskrat population structure reflected watershed network, which may highlight the opportunistic behavior of this species in the use of terrestrial landscape for movements. Muskrat may be considered generalist in their use of the landscape for dispersal. Zalewski et al. (2009) showed that population genetic structure of American mink (*Neovison vison*) did not reflect the watershed structure and that gene flow was not influenced by connection between waterways. We observed similar results in muskrats, indicating the high dispersal ability of these semi-aquatic species and their capacity to use terrestrial corridors for dispersal. Different dispersal strategies are predicted in generalist species as opposed to specialist ones. Habitat specialists may be

more affected by landscape structure than generalists; however Centeno-Cuadros et al. (2011) have demonstrated that the southern water vole, although considered a habitat specialist, uses a wide variety of landscape types during dispersal, thus increasing gene flow.

Cotner and Schooley (2011) found that muskrat were more abundant in urban areas, and considered the muskrat as an urban adapter. The presence of numerous water bodies (approximately 13.5% of the total area) in the urban areas of our study area may explain the high tolerance of muskrats to human activities. As suggested by Cotner and Schooley (2011), these water bodies could be used for dispersal while rivers could be used for house dwelling providing the presence of vegetation as food resources such as cattail (*Typha* sp.). Most studies report on the negative impact of human activities on dispersal due to landscape fragmentation (Keyghobadi 2007; Magle et al. 2010; Fenderson et al. 2011). However human activities can also positively affect gene flow by creating corridors associated with roads (Crispo et al. 2011). Our results showed that including roads in the landscape category of facilitator models for muskrat movement improved the relationship of genetic distance with the LCP models. For the muskrat, ditches and culverts may create movement corridors that can be used to connect optimal habitats thus facilitating dispersal and gene flow. Although roads did not seem to act as a barrier in our study, we have not considered traffic patterns. High traffic levels may have a negative impact on gene flow for example in increasing mortality (Fahrig and Rytwinski 2009). Human activities may also have a positive impact by reducing the risk of predation as predators are generally more affected by human disturbances (Leighton et al. 2010). Finally, in the case of furbearers such as the muskrat, another potential positive effect of urban areas is the lower trapping pressure (Cotner and Schooley 2011).

We found significant patterns using D_{ps} but not a_r. We may have detected significant patterns using D_{ps} because it is a genetic distance that does not require equilibrium assumptions (Bowcock et al. 1994). Moreover, it has been shown that D_{ps} has the power to detect population genetic structure and connectivity at small spatial scale (Murphy et al. 2010). On the other hand, Rousset's genetic distance (a_r) may be considered as the equivalent of Fst-based measures and is more appropriate for examining relationships with historical landscape data (Balkenhol et al. 2009). This may explain why it is less likely to detect significant patterns using Rousset's a_r associated with contemporary landscape data. The choice of resistance cost for the landscape types can affect the results of landscape genetic studies as the relative cost value will change the model sensitivity (Rayfield et al. 2010; Sawyer et al. 2011; Koen et al. 2012). Changing the cost of landscape types did change the least cost path used between individuals but did not change the sensitivity of our relationship. It is possible that our study was conducted at too small a spatial scale to detect differences in genetic structure as the effects of landscape on gene flow are scale dependent. An increase in the size of the study area may allow us to detect the presence of population genetic structure at a larger geographical scale with an effect of landscape characteristics in shaping this structure. The temporal scale may also have an effect on the dynamics of corridors and barriers (Anderson et al. 2010). In several studies, landscape modifications have occurred too recently to affect populations which may explain the lack of detection of population genetic structure (Anderson et al. 2010; Bennett et al. 2010). In our study, the perturbations (urban areas and roads) were well established at the time of study and we do not think that the temporal scale explains the lack of population structure. Future research should increase the spatial scale of study and include other landscape characteristics that may play a role in muskrat dispersal such as substrate

composition, bank height and the width of streams (Cotner and Schooley 2011). Landscape genetic studies of muskrat should also look at the effect of the different types of roads as well as the different traffic levels. We must be careful though not to overstate these results due to our limited sample size (particularly the female sample size), and increasing the number of individuals may help in increasing the power of the results.

In conclusion, population genetic structure of muskrat was not influenced by landscape composition, and landscape features had a limited effect on gene flow. Muskrats had the capacity to use terrestrial pathways between watersheds and roads were not a barrier to movements. On the contrary, it seems that roads may be used as corridors for movements. Our study suggests that semi-aquatic species may be less sensitive to landscape fragmentation than species more restricted to aquatic environments such as amphibians.

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CHAPTER 4. Genetic structure and intraspecific phenotypic variation in muskrat (*Ondatra zibethicus*) populations

Abstract

Extrinsic and intrinsic factors may affect gene flow leading to population genetic structure, and the presence of genetic divergence could ultimately lead to phenotypic differentiation. We examined population genetic structure in muskrat, *Ondatra zibethicus*, across Ontario, Canada, using nine microsatellite loci and two clustering methods (STRUCTURE and TESS). We hypothesized that population genetic structure is present because of major physical barriers and that these genetic differentiations reflect phenotypic divergence. We predicted that muskrat populations would present several genetic clusters across Ontario that would correspond to variation in phenotypic traits. To assess phenotypic differences between the identified genetic clusters, we examined skull morphology using geometric morphometrics. We identified four to five distinct genetic clusters with relatively high levels of gene flow. Muskrat from Manitoulin Island and from southern townships were genetically different from the other regions and from each other, likely as a result of the presence of physical barriers. Some of these genetic clusters such as Manitoulin Island, had distinctly different craniums and mandibles. The differences in skull shape may be to the result of variation in muscles involved in mastication, which in turn may be explained by environmental variation across the study area. The association of genetic divergence with phenotypic variation suggests the effects of genetic drift or local adaptation.

Introduction

Intraspecific genetic variation across different environments is often the result of contemporary and historical processes (Keyghobadi et al. 2005; Gauffre et al. 2008; Pope et al. 2006; Row et al. 2010; Hapeman et al. 2011). The combination of extrinsic (e.g. physical barriers, habitat structure) and intrinsic (e.g. dispersal ability, territoriality) factors as well as historical events affects the extent of gene flow leading to spatial genetic structure. Genetic drift or local adaptation may increase genetic divergence among isolated subpopulations whereas the opposite effect of gene flow will homogenize populations (Lowe et al. 2004; Leinonen et al. 2006; Fernandes et al. 2009). These evolutionary forces affect populations differently across a species' range. However, in the absence of selective pressures, patterns of isolation by distance are often observed in which case there is an increase of genetic differentiation with increasing geographic distances.

The study of intraspecific genetic differentiation and phenotypic variation is critical to better understand evolutionary processes (de Oliveira et al. 2008; Fornel et al. 2010; De Luna et al. 2012). A reduction in gene flow leading to population genetic structuring could lead to population divergence with respect to phenotype, ultimately leading to speciation (Lowe et al. 2004). These phenotypic differences may be the result of local adaptation or genetic drift (Merilä and Crnokrak 2001; Leinonen et al. 2006; Fornel et al. 2010). When local adaptation occurs, heritable phenotypic differences influence individual fitness, leading to selection and genetic changes (e.g. changes in allele frequencies) to a population (Kawecki and Ebert 2004; Hedrick 2005; Smith et al. 2005). However, phenotypic divergence can also be the result of genetic drift in which case there are random changes in the gene pool of small populations (Frankham et al. 2002; Hedrick 2005). Genetic drift can

eventually cause allele fixation leading to a loss of genetic variation (Frankham et al. 2002; Lowe et al. 2004; Spielman et al. 2004; Hedrick 2005). In natural populations, the relative contribution of genetic drift and selection to intraspecific divergence is often unknown. Relationships between intraspecific morphological variation and genetic differentiation have been observed in previous studies for various taxa (de Oliveira et al. 2008; De Luna et al. 2012; Renvoisé et al. 2012) suggesting a role of local adaptation or divergence by genetic drift.

In vertebrates, patterns of phenotypic variation have often been studied using the skull (Monteiro et al. 2003; Lalis et al. 2009; Grieco and Rizk 2010; De Luna et al. 2012), chosen because this morphological structure is complex and may vary as a result of local adaptation reflecting different life history strategies (Monteiro et al. 2003; De Luna et al. 2012). Spatial variation in skull shape has been observed for several species (Monteiro et al 2003; de Oliviera et al. 2008; Lalis et al. 2009; Milenkovic et al. 2010; De Luna et al. 2012) and has largely been found to be the result of differential selection in different environments. Monteiro et al. (2003) detected significant differences in the shape of bones involved in mastication in the punaré rat (*Throchomys apereoides*) because of resource specialization. Significant skull variation was also observed in the harbour porpoise (*Phocoena phocoena*) as a response to different foraging strategies (De Luna et al. 2012).

In this study, we analysed population genetic structure in muskrat (*Ondatra zibethicus*) and examined phenotypic variation among identified populations. The muskrat is a widespread semi-aquatic mammal found in a wide range of habitats including marshes, lakes and streams (Boutin and Birkenholz 1987). This species has a small home range and limited dispersal (Errington 1963; Boutin and Birkenholz 1987; Caley 1987). They feed mainly on available aquatic plants but also eat aquatic invertebrates such as mussels

(Errington 1963; Willner et al. 1980; Boutin and Birkenholz 1987). Geographic variation in skull morphology has been found among muskrat populations in Belgium (Le Boulengé et al. 1996), Finland (Pankakoski and Nurmi 1986), and in Canada between Newfoundland and New Brunswick populations (Rigby and Threlfall 1982).

The purpose of this study was to investigate the population genetic structure of muskrats across Ontario, Canada, and to assess its concordance with phenotypic variation in skull morphology. If population divergence as ascertained by genetic differences is sufficient, then phenotypic divergence should be evident. We hypothesized that population genetic structure would be detected at the large spatial scale of our assessment because of the presence of major physical barriers and that these genetic differentiations would lead to phenotypic divergence. We predicted that muskrat populations would present several genetic clusters across Ontario and that the phenotypic variation observed would match the distribution of genetic clusters.

Methods

Sample collection

A total of 538 muskrats was collected from commercial fur trappers across Ontario during the trapping seasons of 2005-2006 and 2006-2007 (Figure 4.1). Each individual was weighed (±0.01 g), body length was measured (with a tape measure (±0.5 cm)) and sex was determined visually. The head was removed for morphometric analysis and a muscle tissue sample was collected for genotyping. Skulls were cleaned using dermestid beetles at the Royal Ontario Museum (Toronto, Canada) and at Laurentian University (Sudbury, Canada).

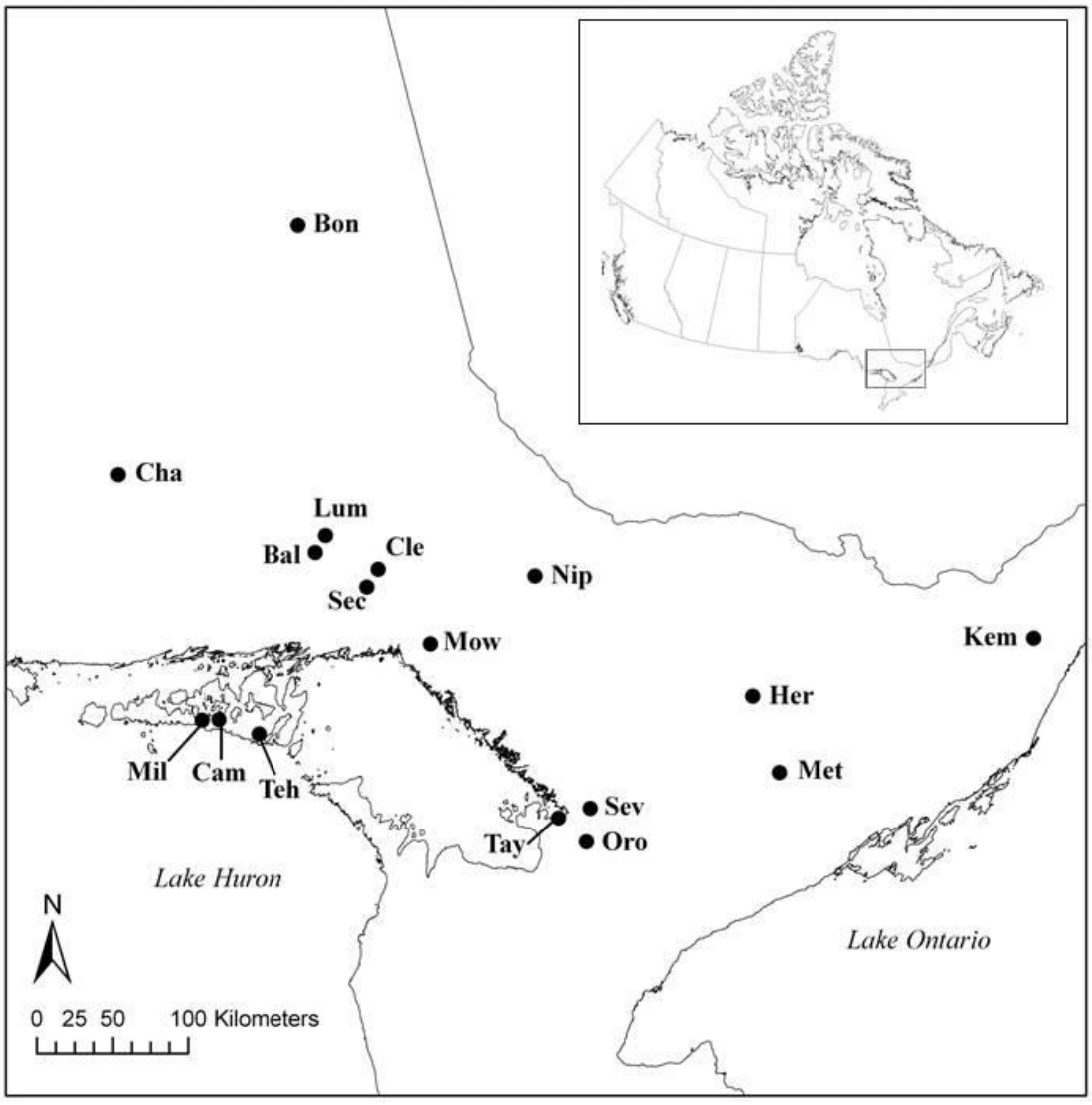

Figure 4.1. Location of the townships in Ontario (Canada) from where muskrats (*Ondatra zibethicus*) were sampled. The full name of the townships are indicated in Table 4.1.

Age was estimated as juvenile (< 1 year old) and adult (> 1 year old) using the first molar index technique (Erb et al. 1999). All specimens were used for genetic analysis however only the adult specimens (N=143) were used for the analysis of phenotypic variation to avoid the confounding effect of age on skull size and shape.

Genetic analysis

DNA was extracted using QIAGEN DNeasy kits following QIAGEN standard procedure for tissue samples. Eleven microsatellite loci (Oz06, Oz08, Oz16, Oz17, Oz22, Oz27, Oz34, Oz41, Oz43, Oz44 and MSCRB5) were amplified following Laurence et al. (2009). PCR products were sequenced at Genome Quebec (Montreal, Canada) on an ABI-3730XL DNA analyzer (Applied Biosystems), and genotypes were scored using Genemarker v2.2.0 (Softgenetics LLC).

Linkage disequilibrium (LD) and deviation from Hardy-Weinberg equilibrium (HWE) were calculated using GENEPOP v.4.1.4 (Rousset 2008). Bonferroni corrections were applied for both LD and HWE (Rice 1989). Scoring errors and the presence of null alleles were assessed using the software MICRO-CHECKER v.2.2.3. (Van Oosterhout et al. 2004) with 95% confidence interval and 5000 randomizations. Genetic diversity was measured by calculating allelic richness (A), observed (H_O) and unbiased expected (H_E) heterozygosities. Allelic richness was calculated using rarefaction in HP-RARE (Kalinowski 2005) to control for unequal sample sizes. Observed and unbiased expected heterozygosities were calculated using GENEPOP v.4.1.4 (Rousset 2008). Differences in allelic richness were analyzed using ANOVA and differences in heterozygosities were estimated using the non-parametric Kruskal Wallis test (Statistica 7, StatSoft, Tulsa, OK).

Genetic structuring of the population was assessed using the programs STRUCTURE v. 2.3.1 (Pritchard et al. 2000) and TESS v.2.3 (Durand et al. 2009). We ran STRUCTURE with ten independent runs per K with K ranging from 1 to 15 assuming admixture and correlated alleles. Each run was conducted with a burn-in of 500,000 followed by 500,000 iterations. For TESS, we ran 50,000 MCMC iterations with 20,000 burn-in for 100 runs with K = 2 to K = 15, under the assumption of admixture. Genetic differentiation between the different townships was also evaluated using F_{ST} (Fstat v. 2.9.3.2; Goudet 2002) and Jost index D (SMOGD; Crawford 2010).

Each animal was provided with a location (township) by the trapper. The exact location of origin for each individual within the township was not provided; therefore we measured the geographical distances using the centroid of the township as the estimated location of each individual. The Euclidean distance between each location was measured with ArcGIS 9 (ESRI). In order to assess isolation by distance, we analyzed the relationship between the Euclidean distance and both F_{ST} and D by performing a Mantel test using the package Ecodist v.1.2.7 (Goslee and Urban 2007) implemented in R 2.13.0 (R Development Core Team 2011).

Phenotypic variation

Three linear measurements were taken on each skull using digital calipers (±0.01 mm): skull length, skull width and interorbital width. These measurements were replicated three times and were found to be highly repeatable (repeated measures ANOVA: $0.0001 < F < 0.058$, $0.810 < p < 0.994$). For each measurement, the average of the three replicates was used for the analyses. We performed a multifactorial ANOVA in Statistica 7 (StatSoft,

Tulsa, OK) to assess the phenotypic variation among the genetic clusters identified by STRUCTURE and TESS for each of the three linear measurements.

To quantify skull shape, each skull was photographed using a digital camera (Olympus Evolt E-300) for the superior view of the cranium, lateral view of the cranium and lateral view of the mandible. Pictures were transformed into tps files using tpsUtil version 1.47 (Rohlf 2010a). Two-dimensional landmarks were assigned for each picture. Landmarks were chosen to capture the shape of the cranium and mandible. These landmarks included 16 landmarks on the superior view of the cranium, 18 landmarks on the lateral view of the cranium and 14 landmarks on the lateral view of the mandible (Figure 4.2, Appendix 1). The coordinates of each landmark were obtained using tpsDig version 2.16 (Rohlf 2010b).

In order to align the specimens and control for size, position and orientation differences, the coordinates were superimposed using Generalized Procrustes Analysis (GPA) (Klingenberg 2010). To quantify the amount of variation between the different genetic clusters, we performed an ANOVA on the Procrustes coordinates. We performed a Canonical Variate Analysis (CVA) using the genetic clusters as classification variables and 10000 permutations to test for pairwise Procrustes distances. All of the geometric morphometric analyses were performed using MorphoJ version 1.05a (Klingenberg 2011).

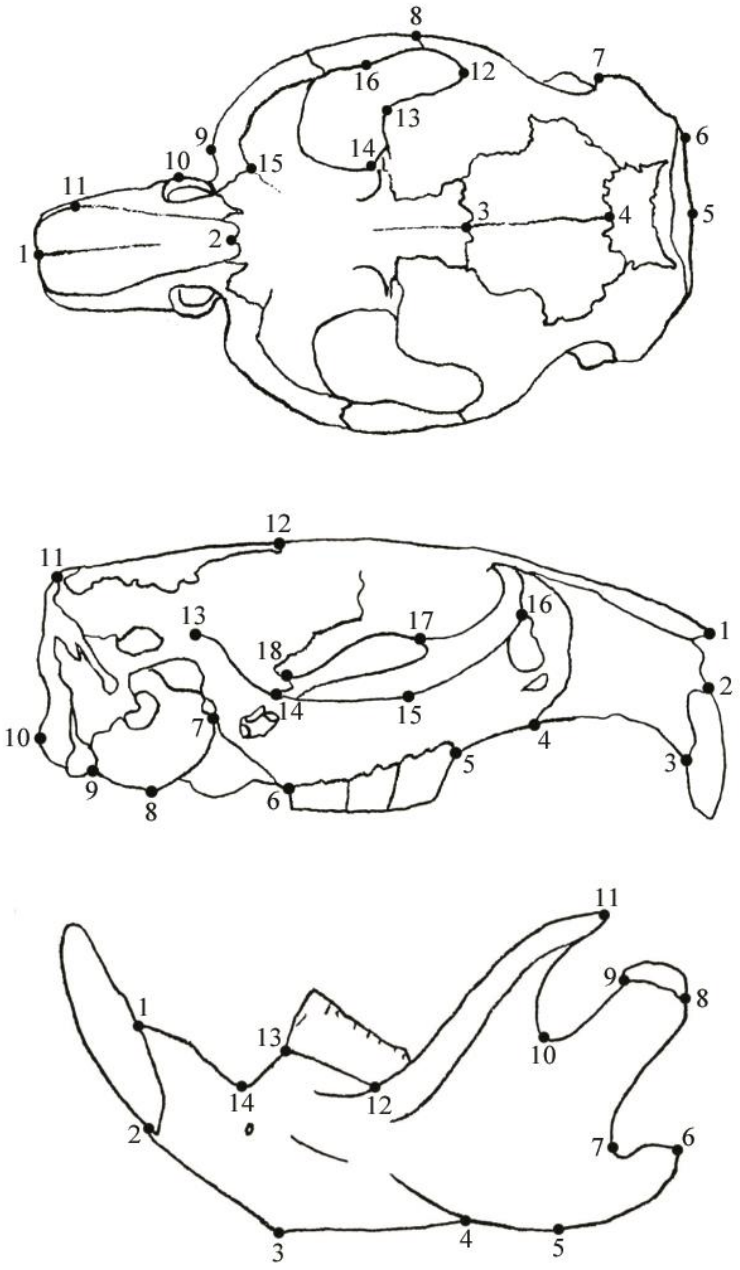

Figure 4.2. Distribution of the landmarks on the (a) superior and (b) lateral views of the cranium and on the (c) lateral view of the mandible of muskrats used in the analysis of phenotypic variation among populations. Definitions of each landmark are indicated in Appendix 1.

Results

Genetic diversity

Two of the eleven microsatellite loci (Oz17 and Oz22) were suspected to have null alleles and were not in HWE in most of the townships. However, we did not detect consistent signs of null alleles and departure from HWE for the remaining loci in the different townships. Therefore, we excluded Oz17 and Oz22 from the analyses and the following results are based on nine microsatellite loci. Linkage disequilibrium was observed for some pairs of loci after Bonferroni correction. However, when present, the pairs showing LD were inconsistent between the townships and therefore we retained the nine loci for all further analyses.

We did not find any significant differences in genetic diversity among the 17 townships in Ontario. Allelic richness ranged from 4.6 to 7.5 ($F_{(16, 136)}$=1.120, p=0.343) and H_o and H_e ranged from 0.68 to 0.86 (H=8.294, p= 0.940) and 0.66 to 0.82 (H=16.196 p=0.440) respectively (Table 4.1).

Genetic structure

Our results from STRUCTURE following the method suggested by Evanno et al. (2005) presented a bimodal curve with peaks at K=2 and K=4 (Figure 4.3a). This may indicate two main clusters with substructure within each of them (Evanno et al. 2005; Ball et al. 2010), which suggests in our case that the most probable K is four. Following Pritchard et al. (2000), mean Ln(P/K) did not plateau but, after K=4, the slope of the curve was not as steep (results not shown). Moreover, the average individual proportion of ancestry (q) was below 0.80 at K higher than 4 (q=0.83 at K=4; Figure 4.3a).

Table 4.1. Genetic diversity of muskrats (*Ondatra zibethicus*) from 17 townships across Ontario (Canada). Number of individuals (N), mean number of alleles per locus (A), observed heterozygosity (H_o) and expected heterozygosity (H_e) are indicated, with standard error in parentheses.

Township	Township name	N	A	H_o	H_e
Bon	Bond	39	7.2	0.80(0.04)	0.81(0.03)
Cha	Chapleau	13	7.5	0.79(0.06)	0.82(0.04)
Mil	Mills	18	5.7	0.83(0.05)	0.75(0.03)
Cam	Campbell	10	4.6	0.68(0.08)	0.66(0.05)
Teh	Tehkummah	37	5.9	0.77(0.05)	0.79(0.03)
Lum	Lumbsden	21	7.1	0.86(0.04)	0.81(0.03)
Bal	Balfour	39	6.2	0.80(0.05)	0.79(0.04)
Cle	Cleland	15	7.0	0.84(0.04)	0.80(0.03)
Sec	Secord	12	6.6	0.85(0.04)	0.80(0.04)
Mow	Mowat	15	6.6	0.84(0.06)	0.75(0.06)
Nip	Nipissing	23	6.7	0.74(0.07)	0.76(0.06)
Tay	Tay	29	6.2	0.77(0.06)	0.74(0.07)
Oro	Oro-Medonte	19	6.9	0.81(0.06)	0.77(0.06)
Sev	Severn	58	7.0	0.80(0.05)	0.79(0.05)
Her	Hershel	27	6.7	0.77(0.06)	0.79(0.03)
Met	Methuen	106	7.3	0.81(0.05)	0.82(0.04)
Kem	Kemptville	22	7.1	0.79(0.06)	0.77(0.06)

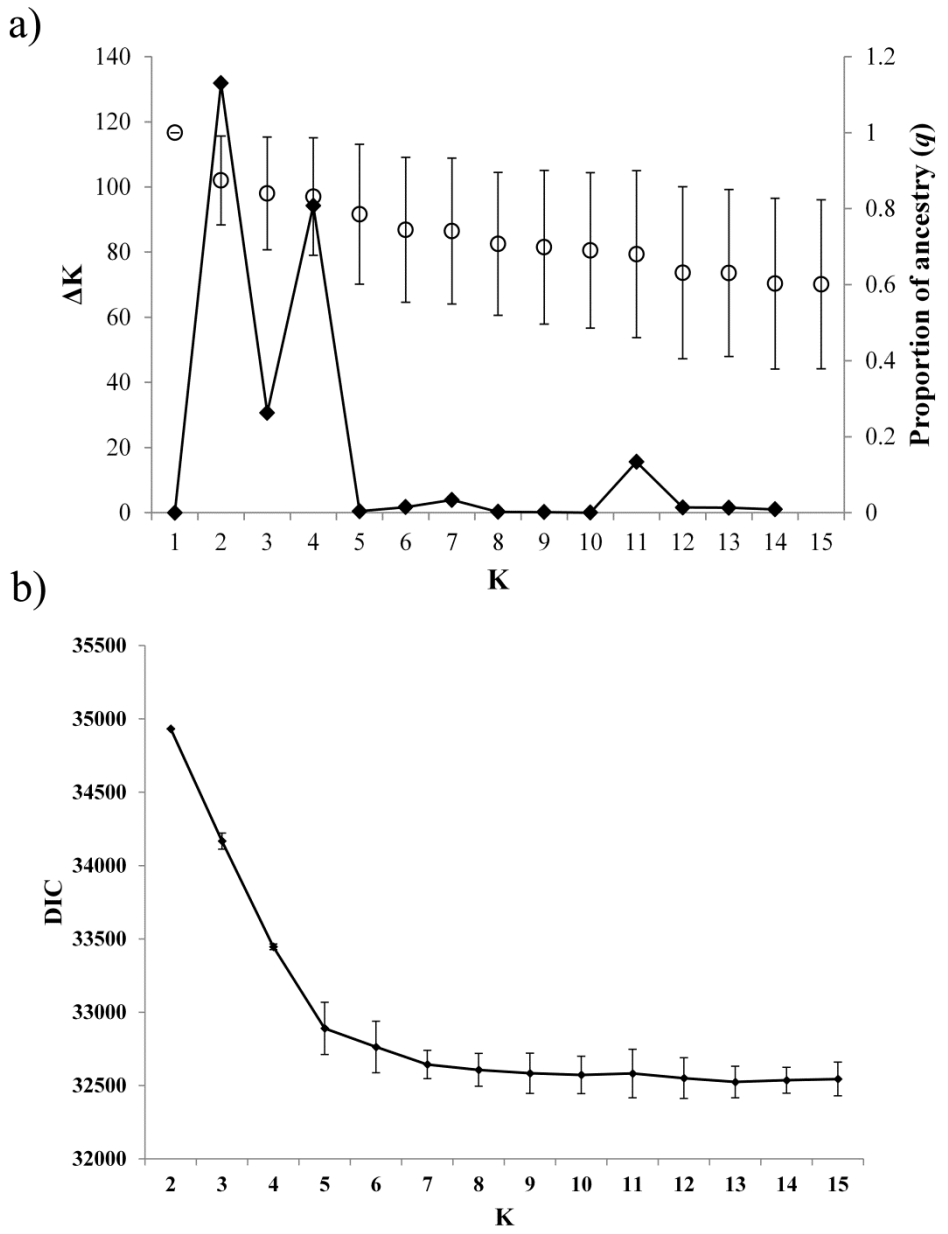

Figure 4.3. Genetic clustering results a) from STRUCTURE v. 2.3.1 with K=1 to 15 showing ΔK (black diamonds) as suggested by Evanno et al. (2005) and average individual proportion of ancestry (q) (open circles), b) DIC values for K=2 to K=15 obtained from TESS v.2.3.

These four clusters corresponded to the northern-western townships (Bon, Cha, Lum, Bal), the townships from Manitoulin island (Cam, Mil, Teh), the eastern townships (Her, Met, Kem), and the southern townships (Sev, Oro, Tay). The remaining townships (Cle, Sec, Mow, Nip) had individuals assigned to both the northern-western and the eastern townships. The estimation of the number of genetic populations using TESS suggested that the most probable K is 5 (Figure 4.3b). The distribution of these five populations is very similar to the one found in STRUCTURE with the exception of the northern-western townships being broken down into two populations (Bon forming one population and Cha, Lum, Bal forming the other one). We did not detect evidence for isolation by distance using Fst (Mantel $r = 0.116$, $p = 0.191$) or using Jost's D (Mantel $r = 0.212$, $p = 0.076$).

Morphometric analysis

No significant differences in skull morphology were detected using the three linear measurements among the genetic clusters obtained from STRUCTURE (Length, $F = 1.466$ $p = 0.231$; ZW, $F = 0.271$ $p = 0.846$; IW, $F = 2.792$ $p = 0.046$, post hoc Scheffé $p>0.05$) or among the genetic clusters obtained from TESS (Length, $F = 2.253$ $p = 0.072$; ZW, $F = 2.306$ $p = 0.067$; IW, $F = 1.340$ $p = 0.264$).

After performing a procrustes ANOVA for each view of the skull using the genetic clusters as a grouping factor, and with sex as an additional factor, we found significant differences in skull size and shape among the clusters. Using both STRUCTURE and TESS clusters, our results indicated significant shape variation among the genetic clusters ($1.32 < F < 1.55$, $p<0.0001$; Table 4.2). The results using the clusters from STRUCTURE also indicated significant variation in size for the superior view only ($F = 2.30$ $p = 0.043$; Table 4.2). We also observed a significant variation in size for the mandible only using the clusters from TESS ($F=2.72$ $p=0.013$; Table 4.2).

In the Canonical Variate Analysis, several Procrustes distances among the genetic clusters were found to be significantly different for clusters from both the STRUCTURE (Table 4.3) and TESS analyses (Table 4.4). Although the differences were not consistent among the three views and between the two approaches for defining clusters, two general patterns were evident. First, the cluster from Manitoulin Island (Cam, Mil, Teh) was regularly observed to be significantly different from the other genetic clusters for all three views of the skull and for both genetic clustering analyses. Additionally, the genetic cluster including the northern-western townships was not different from the genetic cluster including the eastern townships for any of the skull views and for both genetic clustering analyses.

Table 4.2. Results from the procrustes ANOVA for each skull view using the genetic clusters (STRUCTURE and TESS) as a grouping factor with sex as an additional factor.

Skull views		STRUCTURE	TESS
Superior view	Size	F=2.30, p=0.043	F=1.95, p=0.066
	Shape	F=1.55, p<0.0001	F=1.32, p=0.002
Lateral view	Size	F=1.29, p=0.274	F=1.69, p=0.117
	Shape	F=1.47, p<0.0001	F=1.61, p<0.0001
Mandible lateral view	Size	F=0.89, p=0.505	F=2.72, p=0.013
	Shape	F=1.51, p=0.0002	F=1.38, p=0.001

Table 4.3. Pairwise Procrustes distances among the genetic clusters from STRUCTURE for the three skull views from the canonical variate analysis using 10,000 permutations. Procrustes distances are indicated below the diagonal and p-values are indicated above the diagonal. Significant p-values are indicated in bold.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1		Superior 0.664	**Superior 0.007**	Superior 0.058
		Lateral 0.071	**Lateral 0.007**	**Lateral 0.002**
		Mandible 0.245	Mandible 0.168	**Mandible 0.016**
Cluster 2	Superior 0.008		**Superior 0.019**	**Superior 0.011**
	Lateral 0.011		**Lateral 0.006**	**Lateral 0.020**
	Mandible 0.013		Mandible 0.097	**Mandible 0.012**
Cluster 3	Superior 0.016	Superior 0.014		**Superior 0.005**
	Lateral 0.014	Lateral 0.015		**Lateral 0.006**
	Mandible 0.014	Mandible 0.015		Mandible 0.376
Cluster 4	Superior 0.014	Superior 0.015	Superior 0.019	
	Lateral 0.017	Lateral 0.016	Lateral 0.017	
	Mandible 0.022	Mandible 0.022	Mandible 0.015	

Table 4.4. Pairwise Procrustes distances among the genetic clusters from TESS for the three skull views from the canonical variate analysis using 10,000 permutations. Procrustes distances are indicated below the diagonal and p-values are indicated above the diagonal. Significant p-values are indicated in bold.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Cluster 1		**Superior 0.016**	**Superior 0.004**	**Superior 0.028**	**Superior 0.006**
		Lateral 0.021	**Lateral 0.001**	Lateral 0.063	**Lateral 0.020**
		Mandible 0.014	Mandible 0.192	**Mandible 0.012**	Mandible 0.259
Cluster 2	Superior 0.017		Superior 0.218	Superior 0.253	Superior 0.156
	Lateral 0.016		**Lateral 0.002**	Lateral 0.061	**Lateral 0.005**
	Mandible 0.023		Mandible 0.089	Mandible 0.530	Mandible 0.257
Cluster 3	Superior 0.023	Superior 0.015		**Superior 0.006**	Superior 0.112
	Lateral 0.028	Lateral 0.024		**Lateral 0.003**	**Lateral 0.001**
	Mandible 0.025	Mandible 0.031		**Mandible 0.012**	Mandible 0.089
Cluster 4	Superior 0.016	Superior 0.011	Superior 0.020		**Superior 0.015**
	Lateral 0.016	Lateral 0.014	Lateral 0.024		**Lateral 0.0001**
	Mandible 0.022	Mandible 0.013	Mandible 0.033		Mandible 0.192
Cluster 5	Superior 0.020	Superior 0.015	Superior 0.018	Superior 0.017	
	Lateral 0.015	Lateral 0.014	Lateral 0.021	Lateral 0.018	
	Mandible 0.016	Mandible 0.015	Mandible 0.028	Mandible 0.016	

Discussion

The results of this study revealed the presence of population genetic structure of muskrat across Ontario with the presence of four to five genetic clusters. Despite the limited dispersal ability of this species and small home ranges (Errington 1963; Boutin and Birkenholz 1987; Caley 1987), we did not detect strong population genetic structure at the large spatial scale of our assessment. This low level of genetic structuring is associated with high levels of gene flow particularly between the western and eastern regions of Ontario. We did find significant phenotypic variation among some of the putative populations across Ontario; however, contrary to our prediction, the morphological differences observed did not correspond to the genetic clusters identified. The main difference regularly identified (for the different skull views and the different genetic approaches) was between the mainland and Manitoulin Island. The lack of differences between populations in the eastern and western regions was also consistent in all analyses.

Although the level of gene flow was high across our study area, we still detected several genetic clusters that indicate the presence of potential barriers, mainly between Manitoulin Island and the mainland as well as between the southern area and the northern regions. The barrier to gene flow between Manitoulin Island and the mainland may be explained by the large body of water (i.e., Georgian Bay) or by the numerous channels of open water between small islands that muskrats are unlikely to cross. The relatively low level of gene flow between the southern townships and the northern regions may be explained by the presence of a major highway (HWY 400), the southern townships being located on the west side of the highway. Numerous studies report the negative impact of major roads on gene flow (Gerlach and Musolf 2000, Shepard et al. 2008, Holderegger and

Di Giulio 2010) but see Rico et al. (2009) and Crispo et al. (2011). While the detection of several genetic clusters supports our hypothesis of spatial genetic structuring resulting from the presence of major physical barriers, we still found a relatively small number of genetic clusters at the large spatial scale of our assessment. The overall high level of gene flow may indicate a better dispersal ability than what has been observed in previous studies. The muskrat may be considered a generalist in its use of the landscape for movement (Laurence et al. 2013) which may explain its dispersal capacity and hence high level of gene flow. Gene flow may also be facilitated by the high density of waterbodies in our study area and the fact that muskrat may be using roads (ditches and culverts) as movement corridors (Laurence et al. 2013). Zalewski et al. (2009) reported on the strong effect of elevation as a physical barrier leading to population genetic structure in the American mink (*Neovison vison*). However, they also observed that the American mink was able to use a variety of landscapes between rivers during dispersal which may be due to the high density of rivers in their study area. Moreover, the lack of population structure in some species with limited dispersal may be explained by their large effective population size as was the case for several rodent species (Adams and Hadly 2010; Berthier et al. 2005). Although the effective population size for muskrat is not known in our populations, it is possible that in muskrat, large effective population size may have prevented spatial genetic structuring similarly to what has been detected in the California vole (*Microtus californicus*; Adams and Hadly 2010).

Our morphological analysis suggests that only a few of the genetic clusters identified presented morphological differences; these findings do not support our hypothesis that genetic differentiation would lead to phenotypic divergence in which case at least four genetic clusters would have presented significant morphological differences.

Differences in skull shape are often due to variation in the development of muscles involved in mastication (Kiliaridis 1995; Monteiro et al. 2003, 2005). Variation in feeding habits may explain some of the differences observed in the shape of the skull and mandible (Lalis et al. 2009, De Luna et al. 2012). Kiliaridis (1995) demonstrated that rodents feeding on softer food sources developed smaller mastication muscles which resulted in different bone development. Some of the shape differences we observed were located on the rostrum, the zygomatic arch and the shape and angle of the mandible coronoid process. These regions of the skull and mandible are related to the origin and insertion of muscles involved in mastication. The differences in skull shape we detected among some of the muskrat populations may be the result of different feeding ecologies which may be due to different food sources. Muskrat diets include a variety of semi-aquatic plants, however, it may also include invertebrates such as bivalves (Errington 1963; Sietman et al. 2003; Owen et al. 2011). The diet composition and the proportion of each type of food may explain the differentiation in skull shape between some regions, such as between Manitoulin Island and the mainland. Further study on the variation of muskrat diet will be necessary in order to investigate foraging specialization and its relationship to phenotypic divergence.

The presence of genetic divergence associated with morphological differences observed on Manitoulin Island, may indicate the presence of genetic drift and/or local adaptation. De Luna et al. (2012) detected a correlation between genetic divergence and morphological differentiation in the harbor porpoise (*Phocoena phocoena*) and highlighted the possibility of genetic drift and local adaptation in this species. Similarly, de Oliviera et al. (2008) found evidence of population genetic structure in the South American fur seal (*Arctocephalus australis*) associated with strong morphometric differences. Moreover, small mammals display a short generation time which may explain the more responsive

phenotype observed in some populations (Poroshin et al. 2010). Previous studies have also demonstrated the important role of phenotypic plasticity in intraspecific variation of skull shape (Breno et al. 2011, Renaud et al. 2013). Because of some inconsistencies between the phenotypic differences and genetic divergence across our study area, we cannot exclude the possible effects of phenotypic plasticity. Moreover, we did not find consistent morphological differences between the southern and northern regions despite the presence of two distinct genetic clusters. This lack of phenotypic difference may be due to the presence of a recent barrier (highway) between these regions, which would not have given enough time for the populations to differentiate with respect to skull morphology. The absent or weak phenotypic divergence on the mainland may also be due to a small range of environmental variation across the study area suggesting relatively similar habitat and feeding strategy and therefore similar selection pressures.

Future studies may benefit from a larger spatial scale which may help in detecting more phenotypic divergence by including different characteristics of the habitat. Previous studies have shown morphological differences between muskrats from Newfoundland and New Brunswick (Rigby and Threlfall 1982) as well as genetic differences across Canada (Laurence et al. 2011). A morphological comparison among Canadian provinces may provide stronger evidence of divergence with respect to phenotype. Our study highlighted the concordance of phenotypic difference with genetic divergence in some regions indicating the possible presence of local adaptation. These morphological and genetic variations may be the response to ecological variations in a species with a short generation time.

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CHAPTER 5. Genetic structure of muskrat (*Ondatra zibethicus*) and its concordance with taxonomy in North America

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Abstract

Extrinsic factors such as physical barriers play an important role in shaping population genetic structure. A reduction in gene flow leading to population structuring may ultimately lead to population divergence. These divergent populations are often considered subspecies. Because genetic differentiation may represent differences between subspecies, patterns of genetic structure should reflect subspecies groupings. In this study, we examine the contemporary population genetic structure of muskrat (n=331) and assess the relevance of four geographically-distinct subspecies designations across northern North America using nine microsatellite loci. We predicted that patterns of gene flow and genetic structure would reflect the described subspecies. We found evidence of genetic differentiation between western and eastern regions and muskrats from Newfoundland showed significantly lower genetic diversity than central regions. A strong isolation by distance pattern was also detected within the eastern cluster. Our results did not differentiate *O. z. spatulus* (Northwest) from *O. z. albus* (Central), but they suggest a distinction between *O. z. obscurus* (Newfoundland) and *O. z. zibethicus* (East). This study highlights the need for more phylogenetic studies in order to better understand intraspecific divergence and the genetic characterization of subspecies.

Introduction

The genetic structure of contemporary populations is influenced by both intrinsic and extrinsic factors. Extrinsic factors include historical events such as Pleistocene glaciations, which have been frequently identified as major influences on gene flow and thus patterns of intraspecific genetic structure (Avise 2000; Lee-Yaw et al. 2008; Grill et al. 2009).

Subsequent postglacial colonizations have influenced the geographic distribution of genetic variation of numerous taxa (Durka et al. 2005; Pope et al. 2006; Lee-Yaw et al. 2008; An et al. 2009; Braaker and Heckel 2009). Furthermore, contemporary gene flow and population genetic structure are affected by extrinsic physical barriers such as mountains (Rueness et al. 2003) and bodies of water (Jordan and Snell 2008). Contemporary population structure is also shaped by intrinsic biological factors related to gene flow, such as inherent mobility, the tendency toward philopatry, and sex-biased dispersal (Fraser et al. 2004; Worley et al. 2004). Species with small home range sizes and limited dispersal typically show pronounced population genetic structure (Mossman and Waser 2001; Peakall et al. 2003).

The development of population structure and population differentiation is often a precursor to speciation. Significant genetic differentiation within a population is thus a criterion that has been used to designate subspecies, the lowest taxonomic rank used in systematics. Conceptually, subspecies can be defined as interbreeding populations showing genetic differentiation and are often geographically and morphologically distinct from other populations (Lincoln et al. 1998). Although the taxonomic status of subspecies is debatable (Zink 2004; Phillimore and Owens 2006; Cronin 2007; Patten 2010), it is still widely used (Zink 2004; Phillimore and Owens 2006). Subspecies designations are sometimes viewed as arbitrary, but these biological units can represent intraspecific geographic variations and may be relevant for the conservation of threatened and endangered organisms as well as for understanding the evolutionary history of a species (Johnsen et al. 2006; Phillimore and Owens 2006; Winker 2010). Although it is mainly accepted that subspecies should show some level of genetic divergence, traditional subspecies designations were primarily based on morphological differentiation. However, because the phenotype is the result of a combination of genetic and environmental effects, morphological differences may also be

the result of phenotypic plasticity (Crispo 2008; Pfennig et al. 2010). More recent molecular techniques have helped in the genetic characterization of subspecies and the assessment of their relevance (Johnsen et al. 2006; Hull et al. 2008; Grill et al. 2009). Microsatellite loci have been used to infer phylogenies and resolve evolutionary history uncertainties (Richard and Thorpe 2001). The investigation of both intraspecific phylogeography and subspecies resolution is common in ornithology (Hull et al. 2008; An et al. 2009). Although phylogeographic studies are common, few studies have examined both phylogeography and the relevance of subspecies classification in mammals (but see Cullingham et al. 2008; Latch et al. 2009). The subspecific status of mammal species has rarely been assessed unless these species are of conservation concern (Ramey et al. 2005; Grill et al. 2009).

The muskrat (*Ondatra zibethicus*) is a semiaquatic rodent species with a broad geographical distribution across North America. It has been introduced in Europe where it is regarded as an invasive species (Zachos et al. 2007). Although the muskrat is considered an important species in wetland ecosystems (Danell 1996) and a pest with high success of colonization in Europe (Danell 1996; Zachos et al. 2007), population geneticists have largely neglected this species. We only know of one study that looked at population differentiation in Europe using mitochondrial control region sequences (Zachos et al. 2007). Muskrats have small home ranges (Boutin and Birkenholz 1987; Nadeau et al. 1995), they are dependent on watersheds for their food supply and burrows (Willner et al. 1980; Boutin and Birkenholz 1987), and their dispersal abilities are not well known. This restriction to the aquatic environment combined with small home ranges may limit gene flow between populations and lead to genetic structure. Moreover, across North America, natural barriers such as mountains and large bodies of water may act as a barrier to

dispersal for this small semiaquatic mammal. There are currently 16 subspecies of muskrat that have been described across North America, of which 5 are present in Canada (Willner et al. 1980). These subspecies were identified based on color and morphological differences (Boyce 1978; Lewis and Johnson 2002). Variation in skull morphology has also been reported between an island population (Newfoundland [NF]) corresponding to the subspecies *O. z. obscurus* and a mainland population (New Brunswick [NB]) corresponding to *O. z. zibethicus* (Rigby and Threlfall 1982). Nonetheless, no extended geographical comparison study has been conducted, and no genetic assessment of this classification has yet been undertaken. The information available concerning variations in muskrat populations is very limited, and here we used these subspecies designations as a null model to assess the presence of geographic variations.

In this study, we examine the contemporary population genetic structure and assess the relevance of the subspecific status of muskrat using microsatellite DNA loci. We also examined genetic diversity among regions. We hypothesized that muskrat populations will show high genetic structure because of the species' biology (small home range; limited dispersal) and predicted that if the described subspecies represent genetically distinct subgroups, then genetic structure should correspond to existing subspecies designations. We predicted that within the subspecies range, physical barriers such as the Rocky Mountains and large bodies of water would prevent gene flow and isolate populations (e.g., British Columbia [BC], NF, and Prince Edward Island [PEI]).

Methods

Sample collection

A total of 331 tissue samples were collected for DNA extraction. 291 skin samples were obtained from pelts at the Fur Harvesters Auction Inc., North Bay, Ontario, Canada. These samples were collected from 10 different regions (excluding Ontario) across North America (Figure 5.1; Table 5.1). These animals were trapped between October 2006 and March 2007. Additional tissue samples from Ontario (n=40) were collected from muskrat carcasses obtained directly from trappers in October and November 2005. Four of the sixteen subspecies were represented within our samples: *O.z. spatulus* (n=71), *O.z. albus* (n=30), *O.z. zibethicus* (n=205) and *O.z. obscurus* (n=25) (Figure 5.1; Table 5.1). The distribution across North America of the sixteen subspecies is described in Willner et al. (1980).

Genetic analyses

Genomic DNA was extracted using QIAGEN DNeasy tissue kits. Muskrats were genotyped at 12 microsatellite loci (Oz06, Oz08, Oz16, Oz17, Oz22, Oz27, Oz30, Oz32, Oz34, Oz41, Oz43, Oz44) following the procedures described in Laurence et al. (2009). Only nine of these 12 microsatellite loci were used in subsequent analyses because three of them (Oz17, Oz22, Oz30) showed inconsistent peak morphology. Polymerase chain reaction (PCR) products were run on an ABI 3730 sequencer and genotypes were scored using GENEMAPPER 4.0 (Applied Biosystems).

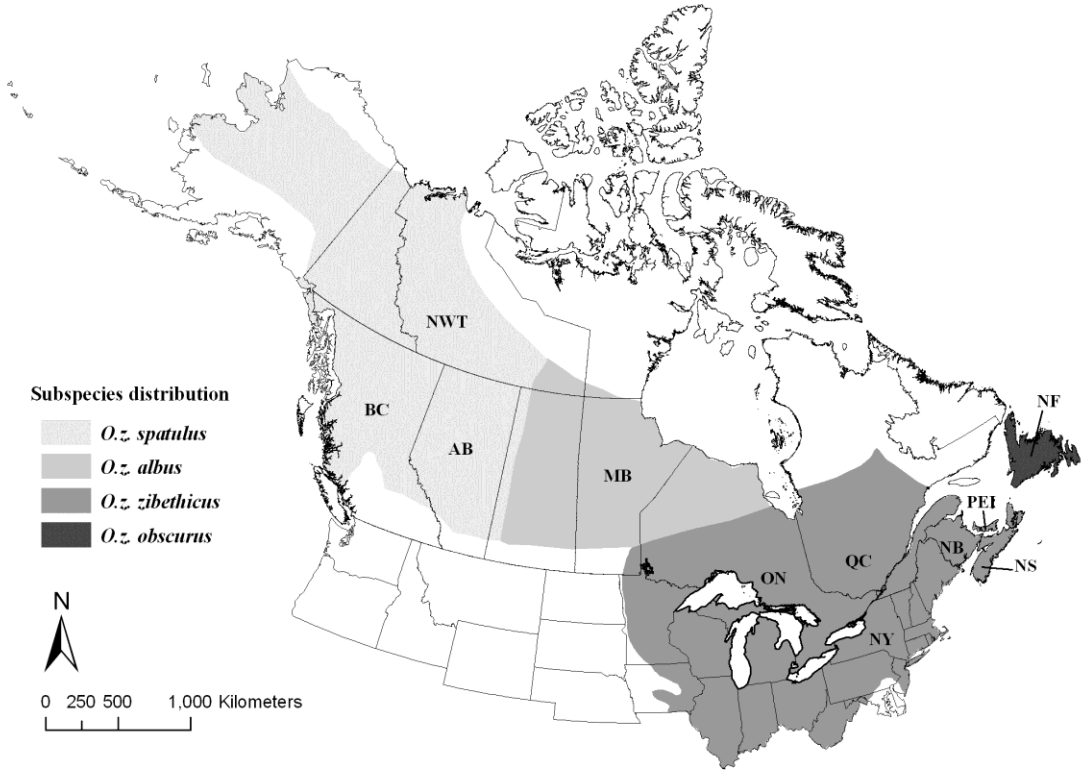

Figure 5.1. Geographic location of the 11 regions sampled and distribution of the four subspecies of muskrat (*Ondatra zibethicus*) studied (from Willner et al. 1980). *BC* British Columbia, *AB* Alberta, *NWT* North West Territories, *MB* Manitoba, *ON* Ontario, *NY* New York State, *QC* Quebec, *NB* New Brunswick, *PEI* Prince Edward Island, *NS* Nova Scotia, *NF* Newfoundland.

Table 5.1. Genetic diversity of muskrat populations across North America. .

Location	Subspecies	N	A (n)	H_o	H_e	Loci not in HWE
BC	*Ondatra zibethicus spatulatus*	11	5.8 (n=11) ^{a,b,c}	0.56±0.05 ^{a,b}	0.65±0.07 ^{a,b}	-
AB	*O. z. spatulatus*	20	6.0 (n=19) ^{a,b,c}	0.62±0.04 ^{a,b}	0.71±0.08 ^{a,b}	Oz43; Oz44
NWT	*O. z. spatulatus*	40	6.5 (n=37) ^{a,b}	0.65±0.03 ^{a,b}	0.67±0.09 ^{a,b}	-
MB	*O. z. albus*	30	7.2 (n=30) ^{a,b}	0.71±0.03 ^{a,b}	0.73±0.07 ^{a,b}	Oz43
ON	*O. z. zibethicus*	44	9.2 (n=43) ^{a}	0.82±0.02 ^{a}	0.85±0.04 ^{a}	Oz06
NY	*O. z. zibethicus*	36	9.3 (n=36) ^{a}	0.83±0.02 ^{a}	0.85±0.04 ^{a}	Oz16; Oz43
Quebec	*O. z. zibethicus*	40	9.0 (n=36) ^{a}	0.82±0.02 ^{a}	0.84±0.05 ^{a}	Oz41
NB	*O. z. zibethicus*	40	8.0 (n=38) ^{a,b}	0.68±0.02 ^{a,b}	0.77±0.08 ^{a,b}	Oz08; Oz16; Oz27
NS	*O. z. zibethicus*	30	4.6 (n=30) ^{b,c}	0.60±0.03 ^{a,b}	0.60±0.08 ^{a,b}	-
PEI	*O. z. zibethicus*	15	4.6 (n=14) ^{b,c}	0.66±0.04 ^{a,b}	0.63±0.09 ^{a,b}	-
NF	*O. z. obscurus*	25	2.8 (n=25) ^{c}	0.36±0.03 ^{b}	0.35±0.09 ^{b}	-

Number of individuals (N) sampled per region and putative subspecies (from Willner et al. 1980). Allelic richness (A), observed heterozygosity (H_o) and unbiased expected heterozygosity (H_e) are indicated with standard error.

n = minimum number of individuals in a sample that were genotyped successfully at all loci

Regions with the same superscript letters did not differ ($p>0.05$; post-hoc multiple comparison tests)

We tested for departure from Hardy-Weinberg equilibrium (HWE) for each region using the software GENEPOP v.4.0.7 (Rousset 2008). Linkage disequilibrium (LD) was tested on all loci using FSTAT v.2.9.3.2. (Goudet 2002). The level of significance for HWE and LD was adjusted by sequential Bonferroni correction to control for multiple tests (Rice 1989). The presence of null alleles and genotyping errors were assessed using the software MICRO-CHECKER v.2.2.3. (van Oosterhout et al. 2004) with a confidence interval of 95% and 5000 randomizations.

Genetic diversity was determined by calculating allelic richness (A), observed (H_o) and unbiased expected (H_e) heterozygosity at each locus. The software HP-Rare (Kalinowski 2005) was used to calculate allelic richness using rarefaction to account for unequal sample sizes. H_o and unbiased H_e were calculated using Fstat v.2.9.3.2. Differences among regions were tested using a one-way ANOVA and Tukey's tests for allelic richness and the nonparametric Kruskal-Wallis and post-hoc multiple comparison tests for H_o and unbiased H_e (Statistica version 6). Pair-wise differentiation (F_{ST}) between regions was determined using FSTAT v.2.9.3.2 (Goudet 2002) and significance of differentiation with Bonferroni correction for multiple tests was tested using 5500 permutations. Pairwise differentiation (F_{ST}) was also determined between the three clusters identified. Centroids of the given state or province were used as reference points to estimate the geographic distances between the different regions as no information other than the province or state of origin were available. We examined the relationship between the natural logarithm of these geographic distances and genetic differentiation (Fst/(1-Fst)) using Mantel tests implemented in FSTAT (10,000 permutations).

Population structure was tested using several approaches. First, we used the Bayesian clustering method implemented by the software STRUCTURE v.2.2 (Pritchard et al.

2000). Five independent runs of each number of subpopulation (*K*) ranging from *K*=1 to *K*=15, were conducted with a burn-in of 1 000 000, followed by 1 000 000 iterations. The runs were performed using correlated allele frequencies model among populations and admixture model. The most probable *K* was estimated by comparing the likelihood (LnP(D)) with the different values of *K* and by selecting the largest ΔK (Evanno et al. 2005) with an average estimated proportion of ancestry for each inferred cluster of 0.80 minimum at that K. This method is not applicable when K=1 (Evanno et al. 2005), therefore, for each round we first looked at the higher estimate of LnP(D) to verify if it was at K=1 (Coulon et al. 2008). This procedure was repeated for each second order cluster until no further subdivision was detected. To assign each individual to a specific cluster, we averaged the q-values across the five runs. Each individual with a $q < 0.6$ were unassigned and left out of subsequent steps (see Coulon et al. 2008).

We also performed a Principal Component Analysis (PCA) using the *adegenet* package in R (Jombart 2008) to visualize the genetic relationships among regions.

Results

Genetic variation

Significant deviations from Hardy Weinberg equilibrium occurred at one to three loci for six out of 11 regions after Bonferroni correction (Table 5.1). No linkage disequilibrium was observed after Bonferroni correction. No evidence of allelic dropout or scoring error due to stuttering was detected. Null alleles may be present based on homozygous excess at two loci (Oz 27 and Oz 44). We performed the analyses without

these two loci and did not detect any differences between the results therefore we maintained these loci in our analyses.

Genetic diversity was significantly different between regions as determined by an ANOVA for allelic richness (A) ($H=45.41$; $p < 0.001$), and a non parametric Kruskal-Wallis test for H_o ($H=29.08$; $p=0.0012$) and H_e ($H=39.43$; $p < 0.001$) (Table 5.1). The island of Newfoundland had the lowest allelic richness and also showed the lowest observed and expected heterozygosity (Table 5.1), whereas central populations were significantly more diverse (ON, NY and QC; post-hoc multiple comparison tests for all three measures of diversity [$0.001< p < 0.013$]) (Table 5.1). Prince Edward Island and Nova Scotia also showed significantly less allelic richness than central populations ($0.003 < p < 0.012$).

Pairwise F_{st} between the regions were all statistically significant ($p\leq 0.05$) and ranged from 0.019 to 0.461 (Table 5.2) with a global F_{st} of 0.167. Newfoundland showed the highest degree of differentiation from the different mainland populations (F_{st}=0.260-0.461). We found a significant positive relationship between geographic distances and genetic distances (Mantel r=0.156; p=0.003) indicating a pattern of isolation by distance.

Table 5.2. Pairwise Fst (from Weir and Cockerham's θ (1984)) between regions (top half of the table).

	BC	AB	NWT	MB	ON	NY	QC	NB	PEI	NS	NF
BC		0.074	0.047	0.063	0.156	0.158	0.173	0.210	0.289	0.326	0.461
AB	*		0.058	0.072	0.152	0.158	0.167	0.207	0.276	0.300	0.420
NWT	**	**		0.041	0.169	0.178	0.184	0.215	0.276	0.298	0.403
MB	**	**	**		0.119	0.126	0.127	0.167	0.243	0.264	0.369
ON	**	**	**	**		0.019	0.026	0.057	0.116	0.157	0.269
NY	**	**	**	**	**		0.019	0.054	0.115	0.156	0.278
QC	**	**	**	**	**	**		0.037	0.113	0.131	0.270
NB	**	**	**	**	**	**	**		0.070	0.082	0.260
PEI	**	**	**	**	**	**	**	**		0.199	0.406
NS	**	**	**	**	**	**	**	**	**		0.342
NF	**	**	**	**	**	**	**	**	**	**	

All values were statistically significant after correction for multiple tests (bottom half of the table; * $p \leq 0.05$; ** $p \leq 0.01$).

Population structure and subspecies status

Following Evanno et al. (2005), the most likely number of clusters suggested by Bayesian analysis implemented in STRUCTURE was K=2 (Figure 5.2a). These genetic clusters corresponded to eastern (ON, NY, QC, NB, PEI, NS, NF) and western (BC, AB, NWT, MB) regions (Figure 5.2b). The average estimated proportion of ancestry for each inferred cluster at that K were high for both eastern and western groups (0.969 and 0.988 respectively). These two clusters were run separately in STRUCTURE until no further subdivision was detected. The eastern group, was subdivided into five clusters (Figure 5.3, Figure 5.4). Most samples from the mainland eastern regions were distributed into different clusters and no clear groupings could be identified on the mainland. Within the eastern region, Newfoundland was identified as a separate cluster. All individuals from Nova Scotia and Prince Edward Island were also assigned to their own cluster but were grouped with thirteen and seven individuals from New Brunswick respectively (Figure 5.3b, Figure 5.4). For the western cluster, LnP(D) was the maximum at K=1 and the average proportion of ancestry was lower than 80% for all K values beyond K=1 (71% for K=2; 63% for K=3 and 55% for K=4). Therefore, we considered that only one cluster was detected for the western regions.

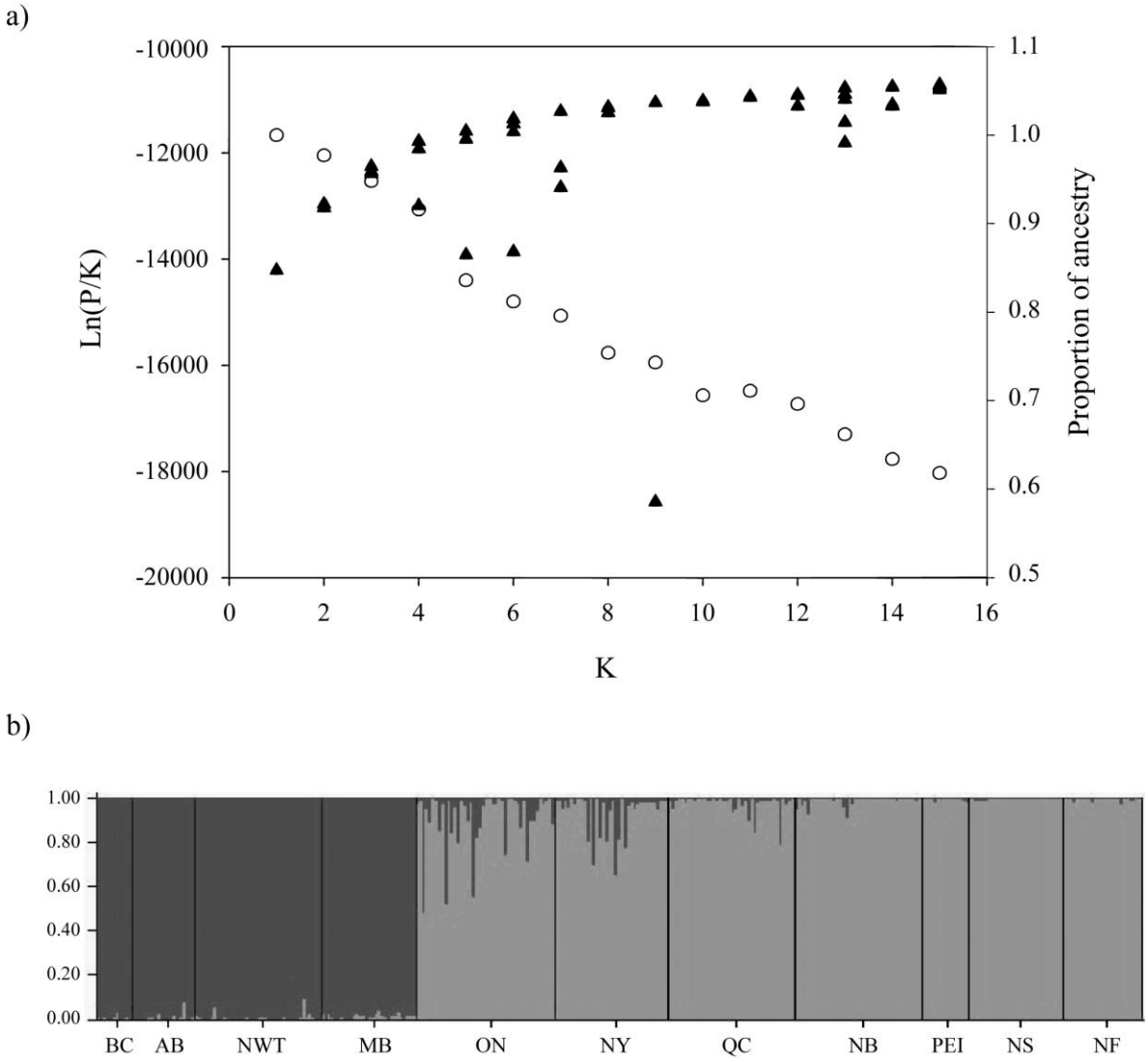

Figure 5.2. (a) Ln(P/K) (▲) and proportion of ancestry (○) using five runs at each K from 1 to 15. (b) Assignment of individuals to each cluster for *K*=2 using STRUCTURE. Individuals are grouped based on their sampling region.

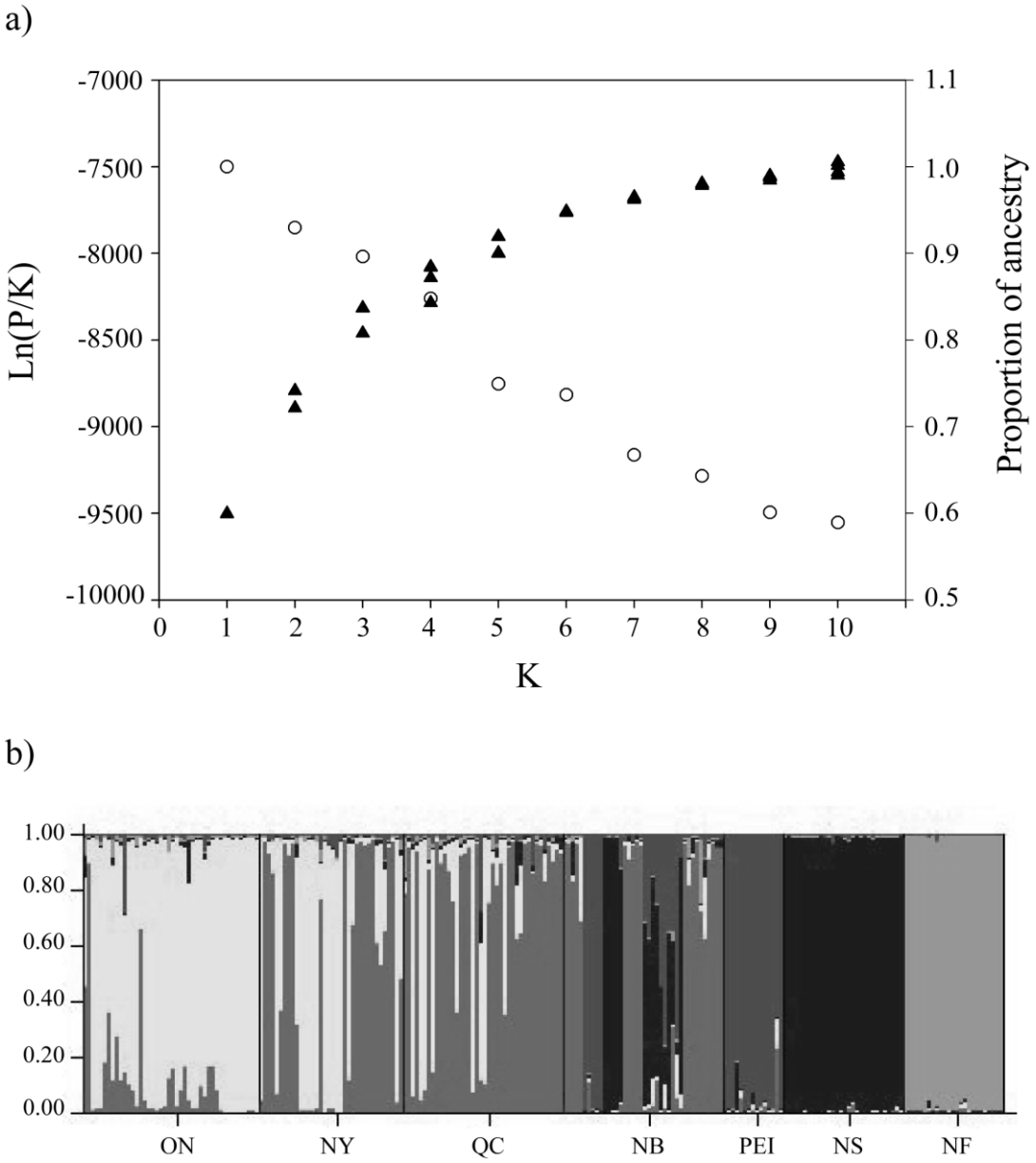

Figure 5.3. (a) Ln(P/K) (▲) and proportion of ancestry (○) for the eastern samples using five runs at each K from 1 to 10. (b) Assignment of individuals to each cluster for *K*=5 using STRUCTURE. Individuals are grouped based on their sampling region.

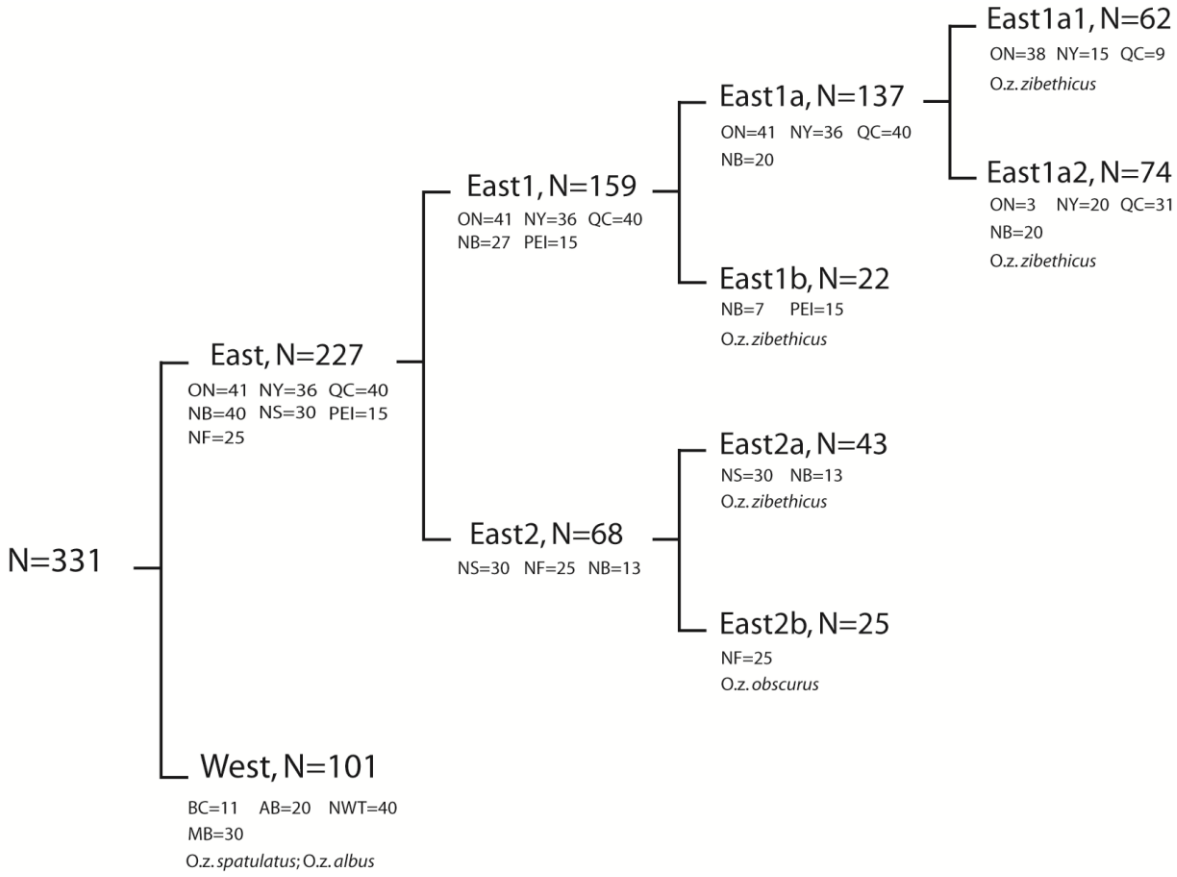

Figure 5.4. Results of Structure analyses for the eastern regions following a hierarchical method. Associated subspecies are indicated for the final clusters.

We were able to identify three distinct clusters using PCA (Figure 5.5). The first principal axis differentiated muskrats from western and eastern regions (eigenvalues for component 1 = 0.616; axis 1 explained 13.6% of the variation), while the second axis further differentiated the eastern regions by separating Newfoundland muskrats from the eastern cluster (eigenvalues component 2 = 0.331; axis 2 explained 7.3% of the variation). The third component (eigenvalue = 0.227; 5% of the variation) did not further differentiate the three clusters. However, when examining the eastern cluster, NS appeared to differentiate from the central regions (Figure 5.5). When examining the eastern regions only, using the PCA (results not shown), we found that NS as well as PEI were differentiated from the central regions on the third component (eigenvalues component 3 = 0.183; axis 4 explained 4.4% of the variation).

Our Bayesian clustering analysis grouped the two subspecies *O.z. spatulus* and *O.z. albus* within the western cluster (Figure 5.4). Within the five clusters identified for the eastern regions, one of them consisted of the *O.z. obscurus* samples whereas the four other clusters corresponded to *O.z. zibethicus* (Figure 5.4). The PCA analysis showed a similar clustering pattern of the four subspecies. The pairwise Fst between the three genetically defined clusters were all statistically significant ($p \leq 0.05$) with Fst between West/East = 0.146, Fst between East/Newfoundland = 0.215 and Fst between West/Newfoundland = 0.343.

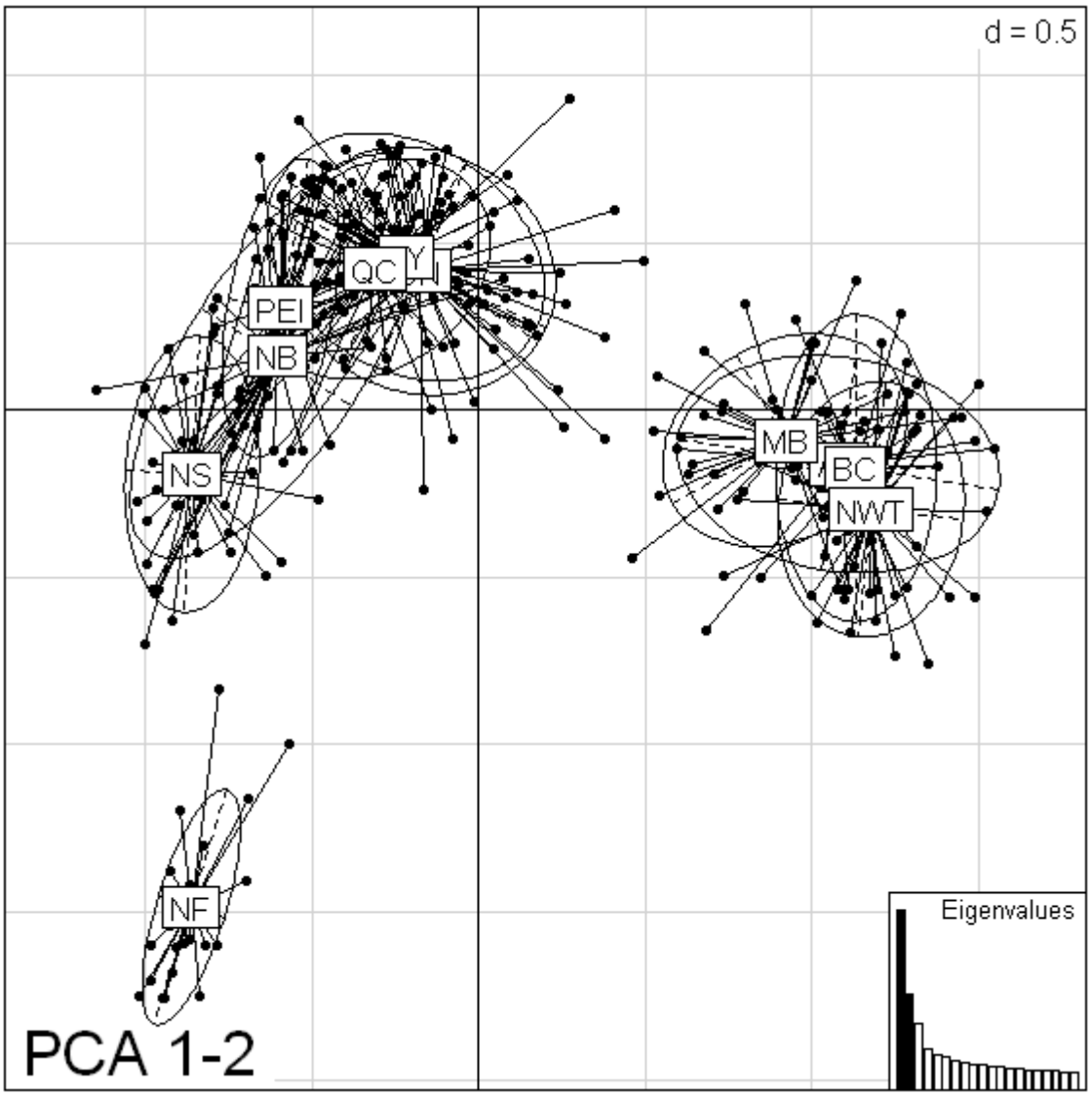

Figure 5.5. PCA of the first two principal components. Each ellipse groups 95% of the individuals for each of the 11 regions. *BC* British Columbia, *AB* Alberta, *NWT* North West Territories, *MB* Manitoba, *ON* Ontario, *NY* New York State, *QC* Quebec, *NB* New Brunswick, *PEI* Prince Edward Island, *NS* Nova Scotia, *NF* Newfoundland.

Discussion

Genetic diversity and genetic structure of populations

We found evidence of genetic structure of muskrat across northern North America with genetic differentiation between western and eastern regions. Our results also indicate a lower genetic diversity for the Newfoundland samples with a significant differentiation from the mainland. Within the eastern regions, muskrat genetic structure reflects a clinal pattern rather than clustered groupings (hierarchical structure analysis and PCA) which seems to be the result of a strong isolation by distance (IBD) at this broad spatial scale. Our results showed some degree of concordance between the genetic structure of muskrat and the existing subspecies designations. However, one of the subspecies (O.z. *albus*) was undifferentiated.

Genetic diversity in muskrat varies among regions and ranged from low (i.e. Newfoundland) to high (i.e. Ontario). Muskrats from Newfoundland showed significantly lower genetic diversity than central populations. The genetic diversity in this island population may be less than mainland populations due to a founder effect generated by glacial retreat which has often been observed in northern regions (Hewitt 2000). Island populations are also expected to have lower genetic diversity and show strong genetic differentiation from mainland populations because of restricted gene flow due to geographic isolation combined with genetic drift (Jordan and Snell 2008). Another possible explanation for this difference in genetic diversity may be related to the size of the area sampled and therefore to the proportion of the population sampled. However, as this information was not available to us, it is not possible to know if there are any differences in the size of the areas sampled. Although not significant, a trend of lower genetic diversity

was observed in regions such as British Columbia and Nova Scotia. In previous studies, lower genetic diversity at the periphery of species range has also been observed because of lower gene flow in these regions (Schwartz et al. 2003; Eckert et al. 2008). The decrease of genetic variability at the periphery of a species' range may also be related to the smaller effective population size found at the margin of the distribution (Johansson et al. 2006).

The Bayesian clustering analysis distinguished populations from eastern and western Canada. However our results suggested that the eastern cluster is further substructured and Newfoundland is separated from the rest of the eastern regions. These results were supported by the PCA. Genetic differentiation between eastern and western regions may be the result of two scenarios of muskrat's colonization of northern regions after the retreat of the ice sheets. Muskrat's post-glacial colonization may have come from distinct glacial refugia, as it has been observed for several species (Hewitt 2000; Schaschl et al. 2003; Lee-yaw et al. 2008) in which case genetic differentiation must have been maintained through time as a result of low gene flow between these regions. On the other hand, the differentiation between eastern and western regions may also be explained by genetically similar colonizers that have diverged due to low gene flow between these two regions. Postglacial colonization from glacial refugia and signs of past barriers are usually examined using mitochondrial DNA (Avise 2000; Schaschl et al. 2003; Lee-Yaw et al. 2008; Flanders et al. 2009). In contrast, because of their high mutation rate, microsatellite loci (nuclear DNA) have been used to characterize genetic differentiation between populations that are the result of more recent events (Pope et al. 2006; Rowe and Beebee 2007). Additional analyses using mitochondrial DNA may help us to detect the effects of historical processes and therefore help us to better understand post-glacial colonization patterns and gene flow between contemporary populations. Rueness et al. (2003) found

strong genetic differentiation between eastern and western lynx (*Lynx canadensis*) populations across Canada. The authors have attributed this genetic separation to the presence of an invisible barrier between these regions that could be related to differences in climatic conditions (continental vs. Atlantic climates). Hull et al. (2008) have also reported a separation between eastern and western red-shouldered hawk (*Buteo lineatus*) populations in North America due to unsuitable habitat preventing gene flow. The broad scale genetic pattern observed for the muskrat may be the result of limited local dispersal due to a heterogeneous landscape and hence restricted gene flow (Pope et al. 2006) as well as limited dispersal due to behaviour such as philopatry (Worley et al. 2004). Although no clear barrier can be identified for muskrat populations, unsuitable habitat such as variable water levels may increase mortality and limit recruitment (Virgl and Messier 1996) and may lead to reduced dispersal and hence reduced gene flow.

Our results show the presence of an isolation by distance pattern. Bayesian clustering method is not well suited for resolving IBD patterned data (Frantz et al. 2009) as most individuals show mixed membership in multiple groups (Pritchard et al. 2000). Bayesian clustering techniques can detect clusters when IBD alone is present (Frantz et al. 2009) and therefore simulate the effect of barriers. In our study, the IBD pattern observed was based on the geographic distances using the centroids of regions because the exact geographic locations were not available and therefore should be taken with caution. Moreover, the clustering of muskrats from Western regions, Eastern regions and Newfoundland was detected using the PCA. Further sampling with a more precise location of each sample would be necessary in future studies in order to assess if the population structure observed is the result of IBD rather than the effect of barriers to gene flow or a combination of both.

Subspecific status of muskrat

From a taxonomic point of view, our results suggest at least three genetically distinct clusters: a western group including *O. z. spatulus* and *O. z. albus*, an eastern group comprised of *O. z. zibethicus* and Newfoundland comprised of *O. z. obscurus*. Contrary to our predictions, the populations from Manitoba were not clearly differentiated from the more western populations (e.g. Alberta and British Columbia) and we found little support for the distinction between the subspecies *O. z. spatulus* and *O. z. albus* using microsatellite data. This lack of genetic differentiation between populations may indicate common ancestry, high levels of gene flow or the combination of both (Johnsen et al. 2006). If muskrat populations from western regions and from the prairies were two distinct subspecies as suggested by Willner et al. (1980), it is possible that recent gene flow between these regions has reduced the genetic distance between these populations. In concordance with the current subspecific status of muskrat in North America, our results do support the presence of a subspecies in Newfoundland (*O. z. obscurus*) showing a clear genetic differentiation from the mainland eastern cluster (*O. z. zibethicus*). Although we recognize that the use of subspecies is subjective, we use these designations as geographical variations of muskrat populations. These biological units may be useful for conservation programs and, as in the case of a widespread species like the muskrat, these subspecific variations may also be interesting in order to understand the evolutionary history of the species.

A caveat to our conclusions is that some samples could be assigned to other subspecies for the western regions (e.g. NWT could be assigned to O.z. *albus* at its southeastern border). First, this does not affect our conclusion regarding genetic structure of muskrat populations

in Canada because all samples within each western province clustered together. Second, the subspecies names we assigned to each population cluster are arbitrarily based on the most widespread subspecies in the province.

The assessment of the subspecific status of this species would benefit from the analysis of other molecular markers such as mitochondrial DNA. Moreover, the use of neutral markers may not reflect the effects of selective adaptation; selection may be strong enough to overcome gene flow, thus leading to morphological differences despite gene flow (Ballentine and Greenberg 2010; Patten 2010). A diagnosis on the relevance of the described subspecies based only on genetic variations is insufficient and a more thorough analysis of morphological traits would also be necessary (Patten 2010).

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CHAPTER 6. General conclusion

Studying the mechanisms that influence patterns of gene flow and their effect on population genetic structure are essential in order to better understand evolutionary processes. In this thesis, I examined the spatial genetic structuring and patterns of gene flow in a semi-aquatic species, the muskrat, at different spatial scales. I used a combination of population genetics, spatial analysis, morphometrics and phylogeography in order to understand the patterns of genetic diversity and their associated phenotypic variations.

Studying intraspecific genetic variations requires the use of molecular markers, among which microsatellite loci have been shown to be useful in the study of recent genetic changes (Selkoe and Toonen 2006). Limited research involving genetic analysis has been done on muskrat which restricts the availability of molecular markers for this species. Among these studies, the D-loop region of the mitochondrial DNA has been used to investigate founder effects in Europe (Zachos et al. 2007) and DNA fingerprinting was used to examine parentage and reproductive strategy in muskrat (Marinelli et al. 1992; Marinelli et al. 1997). In order to investigate contemporary genetic diversity of muskrat at different spatial scales, I successfully characterized 12 species specific microsatellite loci. Ten of them amplified in other rodent species and may potentially be useful in the study of patterns of genetic diversity and population structure in these species. I also successfully cross-amplified one microsatellite locus out of eight loci developed in other rodent species. Scribner and Pearce *(*2000) noted in their review that the success of cross-amplification decreases when there is an increase in the degree of taxonomic relationship. The results from the cross-amplification in this study confirmed the higher success of amplification in species more closely related to the muskrat (family Muridae as opposed to Zapodidae or Sciuridae; Nowak 1991a, 1991b). The characterization of these microsatellite loci has

allowed me to investigate the population genetic structure and patterns of gene flow of the muskrat at different spatial scales.

In a heterogeneous landscape where human activities are in a constant expansion, understanding how landscape characteristics, including anthropogenic features, may impede gene flow is critical in order to better manage and conserve populations. At a microgeographic scale, I hypothesized that fragmentation limits dispersal and predicted that muskrat should exhibit pronounced population genetic structure due to landscape characteristics including physical barriers and a decrease in habitat connectivity. Contrary to my prediction, the landscape features did not lead to population genetic structure at a fine geographical scale. Muskrats have the ability to use different types of landscape to successfully disperse across the study area and the genetic variation was not greater among the watershed than within. Similarly, the fossorial water vole (*Arvicola terrestris*) has the capacity to use various habitats for movements (Berthier et al. 2005), a characteristic also observed in the American mink in Scotland (Zalewski et al. 2009). Semi-aquatic mammals are dependent on the riparian habitat but they have the capacity to use a large variety of landscape types for movement and are not dependent on the water connectivity for dispersal.

The landscape is rapidly altered by human development and anthropogenic factors often lead to population structuring (Noël et al. 2007; Vandergast et al. 2007; Zellmer and Knowles 2009). In this study, anthropogenic features did not prevent gene flow and did not lead to population genetic structure at a fine geographical scale. Gauffre et al. (2008) have noted that the effects of recent barriers are more difficult to identify for species with large effective population sizes. This may explain why anthropogenic factors are likely not representing strong barriers to gene flow in muskrat populations of my study. However, at

the time of study, roads and human-developed lands were well established in the district of Sudbury and the presence of undetectable recent barriers seems unlikely. Gene flow was not affected by landscape features that are considered a barrier in other species such as roads and muskrats may in fact use ditches and culverts as corridors to movement. These findings highlight the tolerance of this semi-aquatic species to fragmentation and anthropogenic landscape features. Muskrat may be considered as generalist in their use of the landscape for movements. Similarly, Cotner and Schooley (2011) considered muskrat as urban adapters providing sufficient suitable habitat and connection among riparian habitats.

At a larger scale, I hypothesized that habitat heterogeneity affects gene flow and may lead to substantial population genetic structuring which ultimately leads to phenotypic divergence among these populations. In this study, the presence of population genetic structure was detected and four to five genetic clusters were identified. However, despite the limited dispersal ability of this species and small home ranges (Errington 1963; Caley 1987), the level of population genetic structure was lower than predicted at this large spatial scale and was associated with high levels of gene flow between the western and eastern regions of Ontario. The lack of population genetic structure may be explained by the large effective population in species with limited dispersal (Adams and Hadly 2010; Berthier et al. 2005). Muskrat may also have greater dispersal ability than what has been reported in previous studies. Despite the high level of gene flow observed, muskrats from Manitoulin Island and southern townships were genetically different from the other regions. These genetic differences may be due to the presence of physical barriers (large bodies of water and major highway respectively) preventing gene flow between these regions. Contrary to my hypothesis, these genetic divergences were not always associated with phenotypic

variations. However, the results of this study indicate that muskrats from Manitoulin Island have diverged genetically and phenotypically from the mainland. The phenotypic variations observed involved regions of the cranium that are related to muscles of mastication. Variation in feeding habits may explain some of the differences observed in the shape of the skull and mandible (Lalis et al. 2009, De Luna et al. 2012). The association of intraspecific genetic divergence with phenotypic variation has been reported in other vertebrate species (de Oliviera et al. 2008; De Luna et al. 2012) and may be the result of local adaptation or genetic drift.

At a macrogeographic scale, I hypothesized that there would be presence of population genetic structuring and isolation by distance mainly because of geographical distances and presence of physical barriers and that the genetic clusters identified would correspond to the described subspecies. In this chapter, I detected strong patterns of isolation by distance across North America and the presence of population genetic structure between western regions, eastern regions and Newfoundland (NF). Physical barriers such as large bodies of water may explain the genetic differentiation between NF and the mainland however no physical barrier explaining the genetic divergence between the eastern and western regions could be identified. This genetic differentiation may be the result of postglacial colonization from distinct refugia associated with low levels of gene flow between these regions or colonization from genetically similar individuals that have diverged as a result of low gene flow between the eastern and western regions. Further understanding of postglacial colonization patterns will require the use of mitochondrial DNA (Avise 2000; Flanders et al 2009). Although the subspecies designations are debatable and sometimes subjective (Zink 2004; Phillimore and Owens 2006), they may be relevant for conservation purposes and for understanding the evolutionary history of a

species (Phillimore and Owens 2006; Winker 2010). Contrary to the prediction, the subspecies designations did not correspond to the genetic clusters identified. The lack of genetic differentiation between some of the subspecies may be the result of common ancestry or high levels of gene flow (Johnsen et al. 2006). In contrast, the subspecies from NF was genetically different from the mainland and this divergence may be maintained by low levels of gene flow across the large bodies of water separating the two regions. This research highlighted the importance of phylogeography studies in order to better understand intraspecific divergence and to help in the improvement of conservation and management of biological units.

Muskrats follow population density cycles of variable length across Canada (Erb et al. 2000). Cycling populations alternate high density and low density phases. These demographic cycles may be another factor explaining differences in diversity of neutral markers and morphological traits (Wojcik et al. 2006). The level of gene flow may vary temporally depending on the population size (Berthier et al. 2005). Low-density phases may be associated with substantial differentiation whereas high-density phases may be associated with higher level of gene flow (Berthier et al. 2005). The spatial genetic and phenotypic variations observed, or lack thereof, may be the result of these density variations (Berthier et al. 2005; Wojcik et al. 2006) and temporal monitoring is necessary in order to better understand the potential effects of demographic cycle on patterns of genetic diversity at various spatial scales.

Overall, in this study, the muskrat presented high levels of gene flow at the different spatial scales. The results of this research are similar with the findings of Adams and Hadly (2010) who detected high gene flow and no population structure in the California vole (*Microtus californicus*) across spatial scales. This high level of gene flow despite relatively

low dispersal ability may be explained by the large effective population size (Adams and Hadly 2010). The overall high level of gene flow and limited population genetic structure in muskrat populations at different spatial scale as well as the lack of influence of landscape composition on gene flow and population genetic structure may explain in part why this species is a successful invasive species in Europe, despite the high human density and high level of urban development. Understanding the dispersal ability of this species may help to better understand colonization patterns in its native and invasive range and is of critical value for the management and control of invasive species (Zalewski et al. 2009).

Future research

This research provides an understanding on how the distribution of genetic variation varies in a semi-aquatic species at different spatial scales. Landscape features did not affect gene flow at a fine spatial scale, whereas physical barriers such as large bodies of water prevented gene flow and led to population divergence with respect to phenotype at a larger spatial scale.

In this study, a lack of population genetic structure was detected at a fine spatial scale, but the study area included a high density of water bodies. Future research would benefit from studying the intraspecific genetic variations of populations in a more fragmented landscape with a smaller percentage of water surfaces in order to examine further the ability of semi-aquatic species to use terrestrial corridors. Moreover, gene flow was not affected by the presence of roads, and muskrat may use ditches and culverts as dispersal corridors. Roads of different sizes and traffic levels may have an effect on gene flow patterns in some species and more research is needed to better understand the effects of anthropogenic features such as roads on population genetic structure of semi-aquatic

mammals (Riley et al. 2006; Frantz et al. 2010). Moreover, trapping effect on population structure has not been considered in this study. In species of economic importance, such as harvested species, areas with more trapping effort may affect gene flow.

At a larger spatial scale, the genetic divergence and phenotypic differences observed in this study may be the result of past barriers as well as more recent factors. By using microsatellite loci, I focused on the contemporary patterns of gene flow and population genetic structure. Future research would benefit from the addition of different types of molecular markers (e.g. mtDNA) in order to better understand the effect of historical factors such as postglacial colonization on the distribution patterns of genetic diversity and phenotypic variation. Finally, future research needs to consider the temporal scale when investigating patterns of genetic variations across different spatial scales as demographic factors and the rapid changes in landscape characteristics may play a role in shaping population genetic structure and patterns of gene flow and their resulting phenotypic variations.

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Appendix 1

Table A.1. Definitions of the landmarks as seen in Figure 4.2.

Landmark #	Definition
Skull – Superior view	
1	Tip of the nasals at their anterior suture
2	Sagittal suture between frontals and nasals
3	Sagittal suture between frontals and parietals
4	Sagittal suture between parietals and interparietal
5	External upper occipital protuberance
6	Postero-lateral point of the occipital
7	Antero-lateral point of the occipital
8	Lateral point on the suture between jugal and squamosal in the zygomatic arch
9	Anteriormost projection of zygomatic arch
10	Lateralmost point on the rostrum
11	Lateralmost point of the suture between nasals and premaxillaries
12	Posteriormost point of the temporal fossa along the squamosal process
13	Antero-lateral point of the squamosal
14	Most medial point on the infraorbital foramen
15	Point at the suture between the frontal and zygomatic arch in the lacrymal region
16	Most medial point on the jugal process of the zygomatic arch
Skull – Lateral view	
1	Anterior tip of the nasal
2	Anteriormost point of premaxillary
3	Inferiormost point of incisor alveolus
4	Inferiormost point of suture between premaxillary and maxillary
5	Anteriormost point of premolar alveolus
6	Posterior edge of third molar where it emerges from maxillary

7	Anteriormost point of the intersection between maxillary and tympanic bulla
8	Inferiormost point on the tympanic bulla
9	Postero-inferior extremity of intersection between occipital and tympanic bulla
10	Most external point on the occipital condyle
11	Posterior extremity of occipital margin
12	Suture between frontals and parietals
13	Posterior point of insertion of the zygomatic process on the squamosal
14	Inferior point of the suture between the jugal and squamosal in the zygomatic arch
15	Inferiormost point on the curvature of the zygomatic arch
16	Anteriormost point of the zygomatic process in the zygomatic arch
17	Superior point of the suture between the jugal and zygomatic process
18	Superior point of the suture between the jugal and squamosal in the zygomatic arch

Mandible

1	Upper extreme anterior border of incisor alveolus
2	Posterior extremity border of incisor alveolus
3	Posterior extremity of the mandibular symphysis
4	Intersection between mandibular body and masseteric crest
5	Inferiormost point of the angular
6	Tip of the angular process
7	Maximum of curvature between condylar and angular processes
8	Posterior edge of the articular surface of the condylar process
9	Anterior edge of the articular surface of the condylar process
10	Maximum of curvature between the coronoid and condylar processes
11	Tip of the coronoid process
12	Intersection between molar alveolus and coronoid process
13	Anterior edge of the premolar alveolus
14	Extreme of the diastema invagination